Universidade de Aveiro Secção Autónoma de Ciências da Saúde

Ano Lectivo 2010/2011

HELENA ISABEL DA COSTA ANTUNES PIMENTEL

Modulação da *Glycogen Synthase Kinase 3*β na regeneração axonal

Glycogen Synthase Kinase 3β modulation in axonal regeneration

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Doutora Márcia Liz (Pós-Doutorada) do Grupo de Regeneração Nervosa do Instituto de Biologia Molecular e Celular-IBMC,Porto

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palavras-chave

Glycogen Synthase Kinase 3β; lesão condicionante; regeneração axonal; lesão na espinal medula

resumo

A espinal medula de mamíferos adultos não possui capacidade de regeneração ao contrário do nervo periférico lesionado. De forma a compreender os mecanismos que potenciem a regeneração do sistema nervoso central, o modelo de lesão condicionante foi usado. Neste, uma lesão no ramo periférico dos neurónios da raiz dorsal cerca de uma semana antes de uma lesão no ramo central dos mesmos neurónios, promove a regeneração do último. Através de abordagens proteómicas concluiu-se que a Glycogen Synthase Kinase 3β (GSK3β) e proteínas que interagem com esta, estavam diferencialmente regulados após lesão condicionante, explicando possivelmente o maior potencial regenerativo nesta condição. Com este projecto, pretendemos compreender o mecanismo de regulação da GSK3β que leva à regeneração axonal. Primeiro observámos nos neurónios da raiz dorsal, um aumento da fosforilação da Akt (a cinase que inactiva a GSK3β através de fosforilação da S9), um aumento da pGSK3β(S9) (forma inactiva) e diminuição dos níveis de pGSK3β(Y216) (forma activa) após lesão condicionante validando os resultados da proteómica. Relativamente à espinal medula (local de lesão), verificou-se a diminuição de pGSK3β(Y216) após lesão condicionante em comparação com a lesão na espinal medula sugerindo que a GSK3β se encontra inibida após lesão condicionante por modulação deste resíduo. Relativamente aos substratos da GSK3β, na espinal medula a fosforilação do CRMP2 encontra-se diminuída e os níveis de pMAP1b encontram-se aumentados após lesão condicionante. De forma a perceber o papel da fosforilação da GSK3β(Y216) na regeneração axonal tratámos culturas de neurónios da raiz dorsal condicionados com ácido lisofosfatídico, um indutor da fosforilação da GSK3β(Y216), o qual reduziu o efeito de condicionamento. Para além disso, o inibidor VII (inibidor da GSK3) provocou um aumento da extensão axonal em neurónios adultos da raiz dorsal possivelmente por diminuição da fosforilação da GSK3β(Y216). Analisámos também o mecanismo responsável pela modulação da fosforilação da GSK3β(Y216). Os nossos resultados sugerem que a Fyn seja um bom candidato, uma vez que observámos na espinal medula níveis aumentados da forma inactiva da Fyn após lesão condicionante. De forma a determinar o papel da GSK3β *in vivo*, a regeneração axonal foi avaliada utilizando ratinhos GSK3βS9A *knockin* (KI). Tanto as culturas de neurónios da raiz dorsal de ratinhos GSK3βS9A KI não lesionados como após condicionamento, tiveram crescimento axonal semelhante à dos ratinhos *wild type* mostrando que a modulação da GSK3β através de fosforilação da GSK3β(S9) não é necessária para o efeito de condicionamento. Os nossos resultados sugerem que a fosforilação da GSK3β(Y216) tem um papel importante na regeneração axonal apesar de a literatura se focar no mecanismo inibitório da fosforilação da GSK3β(S9). A identificação da importância que a GSK3β possa ter no potenciamento da regeneração axonal após lesão condicionante, pode ser positiva no desenvolvimento de novas terapias para lesões na espinal medula.

keywords

Glycogen Synthase Kinase 3β; conditioning lesion; axonal regeneration; spinal cord injury

abstract

The adult mammalian spinal cord fails to regenerate, contrarily to the injured peripheral nerve. In order to shed light on the mechanisms enabling central nervous system axonal regeneration, the conditioning lesion model was used. In this model, an injury in the peripheral branch of the dorsal root ganglia (DRG), approximately one week prior to an injury in the central branch of the DRG, promotes regeneration of the latter. To identify putative candidates differentially regulated in the DRG following conditioning lesion in comparison to spinal cord injury (SCI) alone, two proteomic approaches were used. From these analysis, Glycogen Synthase Kinase 3β (GSK3β) and interacting proteins, were identified as being differentially regulated following conditioning lesion, possibly explaining the increased regeneration in this condition. In this study we aimed to understand how GSK3β is modulated in order to promote axonal regeneration. We observed in the DRG increased levels of pAkt (the kinase that inactivates GSK3β through S9 phosphorylation), an increase of pGSK3β(S9) (inactive form) and decreased pGSK3β(Y216) (active form) levels in the conditioning lesion model, validating the proteomic results. Concerning the spinal cord injury site, we observed a decrease in pGSK3β(Y216) after conditioning lesion when compared to SCI, suggesting that the GSK3β activity is downregulated through modulation of this residue. Regarding the GSK3β substrates, CRMP2 phoshorylation levels are decreased and MAP1b is increasingly phosphorylated following conditioning lesion when compared with SCI alone in the spinal cord injury site. In order to evaluate the role of GSK3β(Y216) phosphorylation in axonal regeneration we treated conditioned DRG neurons with lysophosphatidic acid, an inducer of Y216 phosphorylation, which reduced the conditioning effect. On the other hand, the GSK3 inhibitor VII increased axon growth of adult DRG neurons possibly through a decrease of GSK3β(Y216) phosphorylation. We also analysed the mechanism responsible for the modulation of GSK3β(Y216) phosphorylation. Our results suggest that Fyn is a good candidate as we observed increased levels of the inactive form of Fyn after conditioning lesion in the spinal cord injury site. To evaluate the role of GSK3β *in vivo*, we assessed axonal regeneration in GSK3βS9A knockin (KI) mice. Both uninjured and conditioned DRG neuronal cultures from GSK3βS9A KI mice had similar neurite outgrowth to cultures performed with wild type mice showing that modulation of GSK3β through GSK3β(S9) phosphorylation is not required for the conditioning effect. In summary, our results suggest that GSK3β(Y216) phosphorylation has an important role in axonal regeneration, though the literature focuses on the inhibitory mechanism of GSK3β(S9) phosphorylation. Determining the role of GSK3β in the promotion of axonal regeneration after conditioning lesion, might impact in the development of new therapies for SCI.

Table of contents

Index of Figures

Index of Tables

List of Acronyms

APC	Adenomatous Polyposis Coli
Arg1	Arginase 1
BDNF	Brain-Derived Neurotrophic Factor
CAM	Cell Adhesion Molecule
cAMP	cyclic AMP
CL	Conditioning Lesion
CNS	Central Nervous System
CREB	cAMP Response Element Binding
CRMP2	Collapsin Response-Mediated Protein 2
CRMP4	Collapsin Response-Mediated Protein 4
CSPG	Chondroitin Sulfate Proteglycan
dbcAMP	Dibutyryl-cAMP
DRG	Dorsal Root Ganglia
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFP	Enhanced Green Fluorescent Protein
Eph	Ephrin
ERK	Extracellular signal-Regulated Kinase
GSK3β	Glycogen Synthase Kinase 3β
KI	Knockin
LPA	Lysophosphatidic Acid
MAG	Myelin-Associated Glycoprotein
MAI	Myelin-Associated Inhibitor
MAP	Microtubule-Associated Protein

MAP1b	Microtubule-Associated Protein 1b
MAPK	Mitogen-Associated Protein Kinase
NGF	Nerve Growth Factor
OMgp	Oligodendrocyte-Myelin glycoprotein
PDE4	Phosphodiesterase 4
PI3K	Phosphoinositide 3-Kinase
PKA	Protein Kinase A
PKB	Protein Kinase B
PNS	Peripheral Nervous System
RAG	Regeneration-Associated Gene
SCI	Spinal Cord Injury
SN	Sciatic Nerve

Introduction

1. Central nervous system (CNS) versus peripheral nervous system (PNS) regeneration: extrinsic and intrinsic factors

Spinal cord injury (SCI) disrupts the connections between the brain and spinal cord, often resulting in the loss of sensory and motor function below the lesion site [1]. Understanding why CNS injured axons cannot regenerate after injury in adult mammals has been a major challenge [2]. The reduced intrinsic growth capacity of adult CNS neurons and the poor environment for axon extension, greatly contribute to the failure of CNS axonal regeneration (Figure 1A) [3], resulting in persistent or permanent disabilities of varying degrees of severity [4]. In fact, the growth capability of CNS neurons to elongate axons is markedly reduced during neuronal maturation [3]. Moreover, multiple models have now demonstrated that besides being a mechanical barrier, the molecular composition of the glial scar that is formed after a lesion in the CNS, as well as the production of inhibitory molecules by astrocytes, contribute for unsuccessful regeneration [5]. Compared to the CNS, peripheral axons can and do regenerate, (Figure 1B) resulting in functional recovery when they succeed in reinnervating their targets [6, 7]. Faster and more extensive Wallerian degeneration in the PNS may account for this relatively successful regeneration [8]. This raises the hope that understanding the mechanisms underlying PNS regeneration may lead to new approaches to enhance repair in refractory neuronal populations [4]. The strategy is to improve the rate at which the axons regenerate, being that in mammalians, the rate of axonal elongation is determined by the rate of slow axonal transport [6].

A B

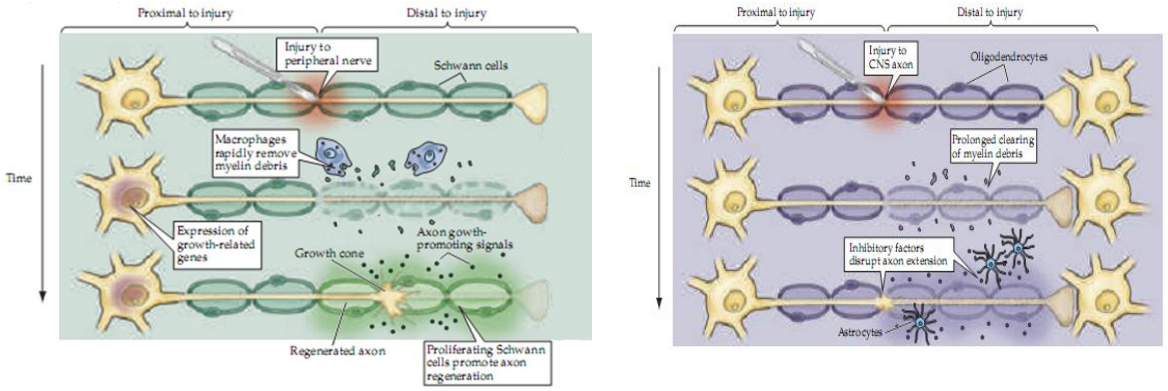


Figure 1: Different responses to injury in the peripheral (A) and central (B) nervous systems. Taken from [9].

1.1. Extrinsic mechanisms

Initial neuronal responses to axon injury in both PNS and CNS are similar in that Wallerian degeneration begins at the distal segment of severed axons, whereas axonal sprouting occurs in the proximal segment. However, the extent and speed of these processes are very different, being that the peripheral axonal regeneration is rather successful compared to that in the CNS. In the PNS, Wallerian degeneration is fast; axon and myelin debris are promptly cleared by Schwann cells and macrophages. This active process seems to be attributable to a steady Na^{+}-dependent extracellular Ca^{2+} entry and is followed by sprouting of the proximal part of axons, resulting in active growth cones at the tip of axons [8]. Schwann cells proliferate to form a Büngner band, that plays an important role in this process, with oriented Schwann cells and extracellular matrix (ECM) guiding regenerative peripheral nerves [10]. Moreover, Schwann cells secrete a variety of neurotrophic and neurotropic factors to support axonal regrowth and guidance [8]. Wallerian degeneration is known to be a prerequisite to ensure normal regeneration following nerve injury [11] as myelin-associated inhibitors (MAIs) including myelin-associated glycoprotein (MAG), oligodendrocyte-myelin glycoprotein (OMgp), Nogo, and netrin1 potently inhibit CNS axonal regeneration. Curiously, MAG is expressed by both CNS oligodendrocytes and PNS Schwann cells [12]. Lack of neurotrophic factors may partly contribute to the regenerative failure in adult CNS [3]. Besides this, the role of

inflammation in CNS repair has been associated with both protective and detrimental effects [13]. Currently there is no direct explanation as to the differential effects exerted by macrophage cells, although it has been proposed that under different conditions these cells may be expressing different phenotypes, which might depend on the timing of macrophage activation [14]. Additionally, dysfunctions of the process of reactive astrogliosis and scar formation have the potential to contribute to, or to be primary causes of, CNS disease mechanisms either through loss of normal functions or through gain of detrimental effects [15]. Traditionally thought to be a simple mechanical barrier, subsequent studies suggested that inhibitory extracellular matrix molecules such as tenascin, semaphorin 3 and chondroitin sulfate proteoglycans (CSPGs), among others are dramatically upregulated during the inflammatory stages after injury. CSPGs generated by astroglial scar around lesions strongly suppress axonal extension via unknown receptor(s) [5]. Axon guidance molecules semaphorin 3A, 5A, and ephrin-3(Eph)B/Eph-A4 may restrict axonal elongation in the injured CNS [3]. Nevertheless, it is important to mention that despite the overall negative connotation of reactive astrogliosis, it exerts numerous essential beneficial functions in neural protection and repair of CNS cells and tissue, in which scar formation lies in the extreme in terms of the severity of responses to CNS insults [15]. Finally, despite the inhibitory environment of the adult CNS, spontaneous regeneration and reorganization of axons are found in animal models of CNS injury. For example, spontaneous axon sprouting of the corticospinal tract is observed proximal to the SCI site. Moreover, a few damaged corticospinal axons can also regrow beyond the lesion site [16].

1.2. Intrinsic mechanisms

Upon embryonic to adult transition, the intrinsic neuronal growth activity is repressed to allow for proper synaptic development, but injury to adult peripheral neurons is capable of reactivating the intrinsic growth capacity. Whether intracellular signaling mechanisms in the growth cones of regenerating axons resemble those in developing nervous system remains largely unknown [8].

Recent studies revealed that simply removing extracellular inhibitory activities is insufficient for promoting successful regeneration in the adult CNS. Importantly, evidence from different species and different models is accumulating to support the notion that diminished intrinsic regenerative ability of mature neurons is a major contributor to regeneration failure [2]. Indeed, removing MAIs or molecules associated with glial scar formation, by genetic deletions or pharmacological inhibitors, only allows limited sprouting [17]. Actually, regrowth involves expression of regeneration-associated genes (RAGs) [2] and in fact in the case of dorsal column lesion, no significant upregulation of RAGs has been observed. On the other hand, the expression of RAGs following peripheral nerve injury is observed [18].

The retrograde transport of injury signals (also called positive injury signals) is one of the essential cellular mechanisms leading to regeneration. Moreover, there is evidence of other injury signals such as injury-induced discharge of axonal potentials and interruption of the normal supply of retrogradely transported target-derived factors (also called negative injury signals). These 3 distinct signaling mechanisms may act in complementary and synergistic roles and coordination between them is necessary to regulate the appropriate genes to promote neuronal survival and increase the intrinsic growth state of injured neurons (Figure 2) [19]. The distinct types of retrograde signals elicited in axons upon injury, range from rapid action potential discharges to slower mechanisms dependent on molecular motors [4]. Rapid ion fluxes at the lesion site are possibly the first indication of damage in axonal integrity upon injury. Lesion causes an increase of Ca^{2+} in the axon which propagates a depolarizing response to the soma via activation of voltage-dependent sodium channels [4]. A transient and localized increase in axonal calcium caused by axotomy, induces localized proteolytic activity which is a necessary step in the cascade of events that leads to growth cone formation [20]. Calcium waves are also likely important for cytoskeletal rearrangement and growth cone formation, as long-range intracellular Ca^{2+} signaling changes motility in migrating neurons by regulating RhoA distribution [4].

In vitro studies showed that electrical stimulation accelerates motor [21] and sensory [22] axon outgrowth and increases cyclic AMP (cAMP) levels in dorsal root ganglia (DRG)

neurons. However, electrical stimulation of CNS does not promote regeneration [23] suggesting that electrical stimulation alone is not sufficient to trigger a complete regenerative response [19]. Thus, rapid electrophysiological mechanisms are likely to play a role in the initial priming of an injured neuron for growth, but effective regeneration requires a second phase of signaling [4].

Work over the past ten years has confirmed that axons can synthesize proteins locally. Indeed, mature neurons use axonal mRNA translation to locally synthesize injury signals that are transported to the nucleus of injured neurons [24]. The positive injury signals identified so far share one common requirement: microtubule-dependent retrograde transport. Combined with a protection mechanism against phosphatases during transport, activation and retrograde transport of mitogen-associated protein kinases (MAPKs) might play an important role in regeneration. In addition to MAPKs, axonal injury activates several transcription factors through the local release of cytokines [19].

Although interruption of normal supply of retrogradely transported cues represents another important mechanism to sense injury, little is known about this type of signaling. Once a neuron is connected with its target, the intrinsic neuronal growth activity must be repressed as above mentioned. In order to allow regeneration to occur, this repression must be released. Nevertheless, future studies are needed to explore the role of negative injury signals in axonal regeneration. Despite the fact that neurotrophins represent the ideal candidates, evidence for their role as negative signals following injury has not yet been established [19]. Importantly, treating neurons overnight with neurotrophins (priming) such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) is another means of stimulating cAMP production, not by inducing cAMP synthesis but instead, preventing its degradation. When neurons are primed, neurotrophins activate Trk receptors and extracellular signal-regulated kinase (Erk) [25] producing a transient inhibition of phosphodiesterase 4 (PDE4) activity, the main responsible for cAMP hydrolysis. This causes cAMP to accumulate until reaching a threshold level, sufficient to override inhibitory signals mediated by MAG, Nogo-A and OMgp when the neurons are subsequently plated on myelin [26]. However, neurotrophins cannot directly overcome inhibition by myelin, making them ineffective as a treatment for SCI. Cell-permeable

cAMP analogues are a more effective way of directly elevating intracellular cAMP levels, being dibutyryl cAMP (dbcAMP) one of the most widely used agents. Administration of dbcAMP alone overcomes inhibition by MAG and myelin. However, targeting downstream effectors of cAMP may be more specific and thus more effective in promoting regeneration. By using specific gene products, it may be possible to directly affect the cytoskeleton while avoiding detrimental side effects such as inflammation [25].

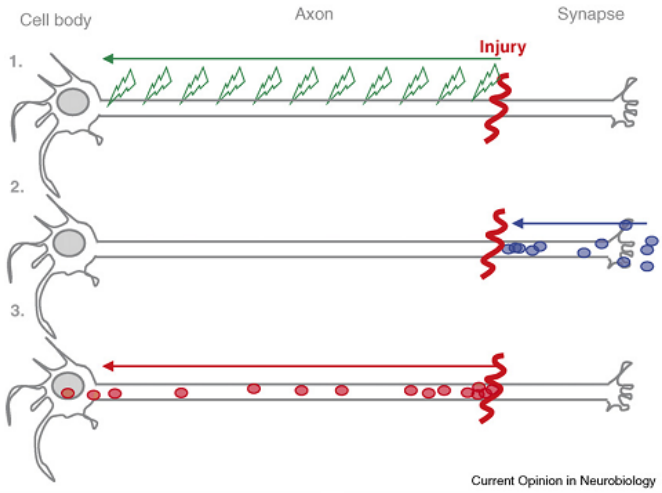


Figure 2: Signaling mechanisms. (1) injury-induced discharge of axonal potentials, (2) interruption of the normal supply of retrogradely transported trophic factors or negative regulators of neuronal growth from the target, and (3) retrograde transport of activated proteins emanating at the injury site, termed positive injury signals. Taken from [19].

2. The conditioning lesion (CL) model in axonal regeneration

Axonal regeneration can be manipulated [6] by intrinsically changing neurons so that they no longer recognize myelin as inhibitory [27]. If the peripheral axonal branches of DRG neurons are injured prior to lesioning the central axonal branch, the neurons are primed into a growth state and regenerate their central branches through a SCI site [4]. It has been described that optimal regeneration is obtained when the peripheral lesion precedes the central lesion by 1 week, extending hundreds of micrometers beyond the site of injury [25]. This paradigm has been described as the conditioning lesion model and correlates with increased gene expression and protein synthesis in the neuronal cell body and an increased rate of slow axonal transport [6]. In fact, retrograde injury signals travel

from the peripheral injury site back to the cell body to increase the intrinsic growth capacity of the neuron, enabling centrally injured neurons to regenerate. The conditioning injury suggests that distinct signaling mechanisms regulate responses to central versus peripheral injury in DRG neurons and may contribute to their different abilities to axonal regrowth [19]. Indeed, in one study all the tested RAGs were upregulated multiple fold in DRG neurons conditioned before the CNS lesion [18]. Interestingly, a similar upregulation was induced in animals that were either conditioned 2, 4, or 8 weeks after CNS lesion or that underwent peripheral lesion only, indicating that the temporal order of the central and peripheral lesions does not affect the degree of upregulation of RAGs. Actually, DRG neurons conditioned after SCI also acquire the ability to grow but only in absence of traumatic tissue [28]. For both pratical and ethical reasons, this finding is extremely valuable, as a priming injury in the peripheral nerve cannot be inflicted in humans to encourage growth of spinal axons [29].

Primary sensory neurons with cell bodies in the DRG are a useful model to study the mechanisms that regulate regeneration. DRG neurons are pseudobipolar neurons and possess 2 axonal branches: a peripheral axon that regenerates when injured and a centrally projecting that does not regenerate following injury. Remarkably, lesion in the peripheral branch before injury to the central branch promotes regeneration of central axons (Figure 3) [19]. The sciatic nerve (SN) is a commonly used model system for regeneration studies, comprising a mixed population of motor and sensory axons. The sensory neurons extending into the SN are located in the lumbar 4 (L4), L5 and L6 DRG that innervate skin and muscle of the hind limb [4].

Two of the conditioning lesion (CL) hallmarks is that the speed at which axons regenerate is manipulated and it raises the cAMP levels in DRG neurons similarly to what occurs in embryonic DRG neurons; a time of intense and rapid axonal growth [6]. Injections of cAMP analogues such as dbcAMP allows the axons to overcome myelin inhibition both *in vitro* and *in vivo,* mimicking the conditioning lesion (CL) effect. However, an important distinction has to be made between these two strategies. Although dbcAMP can overcome CNS inhibition of elongation, it does not increase the intrinsic growth state as conditioning lesion (CL) does [30].

It is now known that the effects of cAMP are initially transcription independent (and protein kinase A (PKA)-dependent), causing the activation of PKA and later they become transcription dependent (and PKA-independent), by activation of cAMP response element binding (CREB) which leads to upregulated expression of genes such as arginase 1 (Arg1) and interleukin-6 whose products have been shown to directly promote axonal regeneration, overcoming myelin inhibition [25]. In particular, overexpression of Arg1 is sufficient to overcome inhibition by MAG [31].

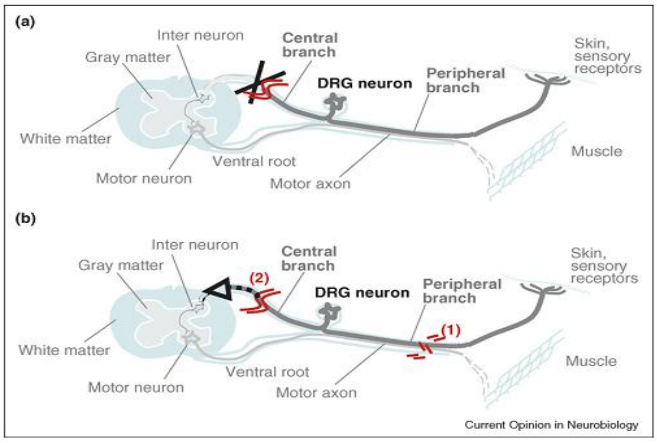


Figure 3: Conditioning injury paradigm. Sensory axons in the adult spinal cord do not regenerate after injury (a), while peripheral injury results in a robust regenerative response. Regeneration of the central branch can be greatly enhanced by a previous injury to the peripheral branch, referred to as a 'conditioning injury' (b). Taken from [19].

3. Cytoskeleton rearrangement

Neuronal growth cones are the machinery for detecting the environment for growth and guidance signals and also the major driving force for axonal elongation [8]. In fact, growth cones guide axons through appropriate routes, to locate a suitable synaptic partner (pathfinding), by activation of receptors for guidance molecules in their plasma membrane [32]. This 'road' comprises adhesive molecules that are either presented on a neighbouring cell surface (for example, transmembrane cell adhesion molecules (CAMs)) or assembled into a dense ECM. Additionally, anti-adhesive surface-bound molecules can prohibit the advance of the growth cone [33]. Finally, these molecules may be

encountered as a concentration gradient that diffuses from an intermediate or final target source, presenting further steering instructions to the travelling growth cone [34]. Contrarily to what was previously thought, it is now known that the response to attraction or repulsion is not due to the intrinsic property of the cue, but instead to the specific receptors and internal signaling milieu of the growth cone [33]. The activation of receptors for guidance molecules in growth-cone plasma membranes drives intracellular signaling pathways that converge mainly at the growth-cone cytoskeleton [35]. Interestingly, the route that the growth cone must take to pathfind is rarely a straight one. Growth cones must make steering manoeuvres to change direction in choice points, and consequently reorganize the growth-cone cytoskeleton [32].

3.1. Structure of the growth cone and axon outgrowth

The structure of the growth cone depicted on Figure 4 is fundamental to its function [33]. The filopodia are the first part of the growth cone coming into contact with guidance molecules and thus having an important role in axon guidance [36]. Filopodia stabilize when they encounter an attractive guidance molecule and retract when on contact with a repellent one [32]. In between the filopodia lie sheets of membrane called lamellipodia-like veils [33]. The growth cone can be separated into three domains based on cytoskeletal distribution [37]. In the peripheral (P) domain, the organization of actin filaments (F-actin) is different in lamellipodia and filopodia. Lamellipodia are composed of branched dendritic actin network, whereas filopodia have parallel bundles of F-actin [32]. Additionally, individual dynamic microtubules explore this region, usually along F-actin bundles [33]. The central (C) domain comprises dynamic microtubules that extend into the P domain in addition to vesicles and numerous organelles, such as mitochondria and reticulum. A subset of microtubules in the C domain entering the growth cone from the axon shaft are relatively stable and generally restricted to this domain [32]. Finally, the transition (T) zone lies at the interface between the P and C domains, where actin arcs (actomyosin contractile structures) locate perpendicular to F-actin bundles [38]. The

dynamics of these cytoskeletal components determine growth cone shape and movement [33].

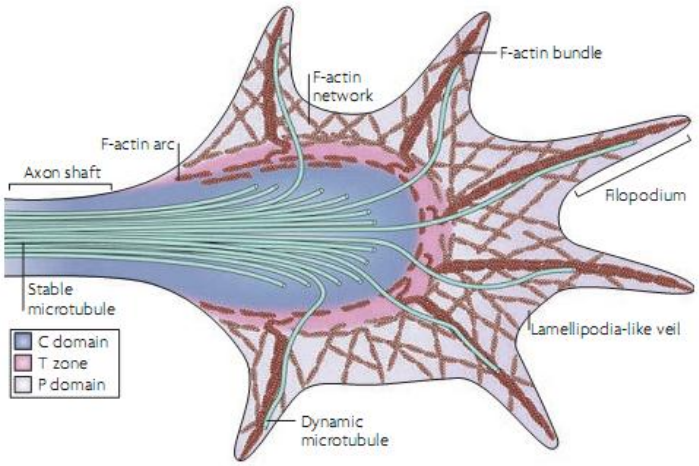


Figure 4: The structure of the growth cone. Taken from [33].

Actually, the growth cone engages its cytoskeleton to progress through three stages: protrusion, engorgement and consolidation [37] (Figure 5). The receptors in the distal end of the growth cone bind to an adhesive substrate, activating intracellular signaling cascades which result in the linkage between the substrate and actin [33]. Protrusion corresponds to the extension of lamellipodia and filopodia at the axon tip through actin filament polymerization which might be regulated by microtubules [39]. During engorgement, F-actin arcs reorientate towards the site of new growth and guide the C domain microtubules to advance and invade protrusions, carrying also membrane vesicles and organelles [33, 39]. Finally, consolidation occurs as the proximal part of the growth cone assumes a cylindrical shape and transport of organelles becomes bidirectional, thus forming a new segment of axon shaft [37]. The myosin II-containing arcs compress the microtubules into the newly localized C domain [33]. These three continuous stages occur during the formation of nascent axons, as well as during axon branching when new growth cones form from an axon shaft [37].

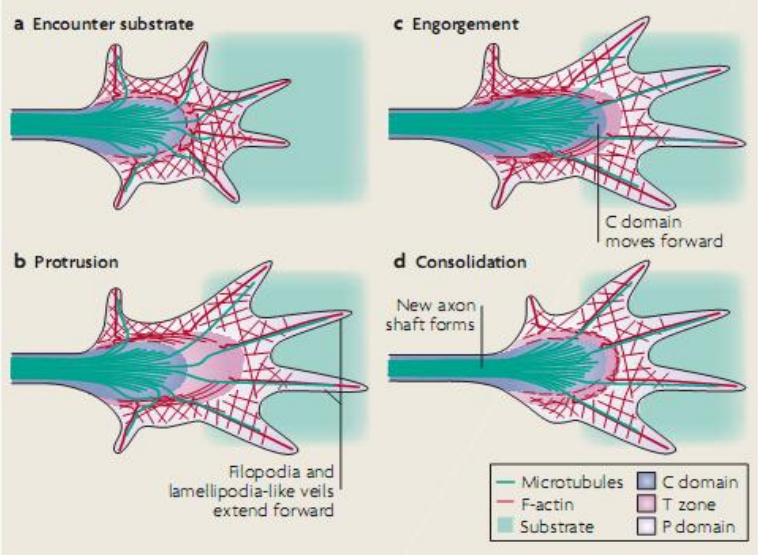


Figure 5: Description of the axon outgrowth stages: protrusion, engorgement and consolidation. Taken from [33].

3.2. The growth-cone cytoskeleton

Two cytoskeletal filaments invariably present in the growth cones are microtubules and actin [32]. The cytoskeleton and associated motor proteins play a critical role in axonal retrograde transport with microtubules being particularly relevant because they provide long-range tracks for transport and are mostly uniformly oriented in axons with plus ends towards the terminals and minus ends towards the cell body [4]. At the plus end, tubulin assembly takes place preferentially. However, there is some evidence for anterograde transport probably mediated by the dynein motor complex [32].

Microtubules are cylindrical polarized structures composed of linear arrays of alternating α- and β-tubulin subunits associated laterally, forming protofilaments [33, 39]. Microtubules are dynamic tubulin polymers that switch between growing and shrinking phases, a process known as dynamic instability, characterized by the rate of polymerization and depolymerization and the frequencies of catastrophes (transitions from polymerization to depolymerization) and rescues (transitions from depolymerization to polymerization) [39]. Several proteins bind to microtubules: some proteins stabilize microtubules (for example, microtubule-associated protein 1B (MAP1b)) [33], some act as microtubule motors (for example, dynein and kinesin) [40] and others are part of a family

called plus-end tracking proteins (+TIPs), which have been implicated in dynamic control of plus end microtubules and linking microtubules with actin- or membrane-associated structures (for example, adenomatous polyposis coli (APC)) [41].

Actin filaments are also polarized, with a barbed end (plus end) pointing towards the cell membrane where monomeric actin is generally assembled near/at the tip of filopodium and a pointed end (minus end) [32]. Their formation, stability and destruction are carefully regulated at every stage in the growth cone [33]. In fact, the plus end addition of globular(G)-actin produces a force that moves the F-actin bundle rearwards designated by retrograde flow. On the other hand, orthograde flow consists on the force on the plasma membrane that extends the filopodium [32]. Which mechanism predominates depends partly on the strength of the adhesion of the filopodium to the substratum where the growth cone is growing on [42].

It is now suggested that there is a physical interaction between F-actin and microtubules. Whether this is a direct interaction or mediated by specific proteins has not been determined [32]. Actually, actin has a pivotal role in determining microtubules localization in the growth cone [43]. Microtubules extend further into P domain when F-actin retrograde flow is attenuated and less successfully when retrograde F-actin flow is increased [32]. Moreover, distinct classes of actin structures seem to regulate different populations of microtubules. Whereas F-actin bundles can inhibit the protrusive activities of P domain microtubules when they are coupled together, F-actin arcs regulate the engorgement and consolidation activities of C domain microtubules [33].

3.3. Microtubules organization and axonal regeneration

After injury, the formation of a growth cone at the tip of a transected axon is crucial for the subsequent axonal regeneration. The tips of lesioned axons located in the PNS transform into growth cones capable of sustained growth. The growth cone efficiently uses membrane trafficking, energy and an intact cytoskeleton for elongation. Stable microtubules are aligned in parallel bundles and serve as tracks for organelle transport to support the rapidly advancing growth cone and to form the backbone of the growing

axon. On the other hand, lesioned CNS axons form characteristic swellings at their tips known as retraction bulbs, which lack a regenerative response [44]. These differences underlie distinct cytoskeleton dynamic rearrangements which are essential for axon outgrowth [37]. Remarkably, *in vivo* growth cones contain bundled dynamic microtubules that reach the axonal tip whereas the dynamic microtubules are mislocalized in retraction bulbs. Frank Bradke and collaborators have shown that retraction bulbs are characterized by accumulated organelles (including trans-Golgi-derived vesicles and mitochondria) and dispersed microtubules compared with unlesioned axons and growth cones. Indeed, they concluded that destabilization of microtubules was sufficient to cause formation of structures reminiscent of retraction bulbs. Contrarily, they observed that stabilization of microtubules precludes the formation of retraction bulbs and is sufficient to induce elongation of CNS axons after lesion [44].

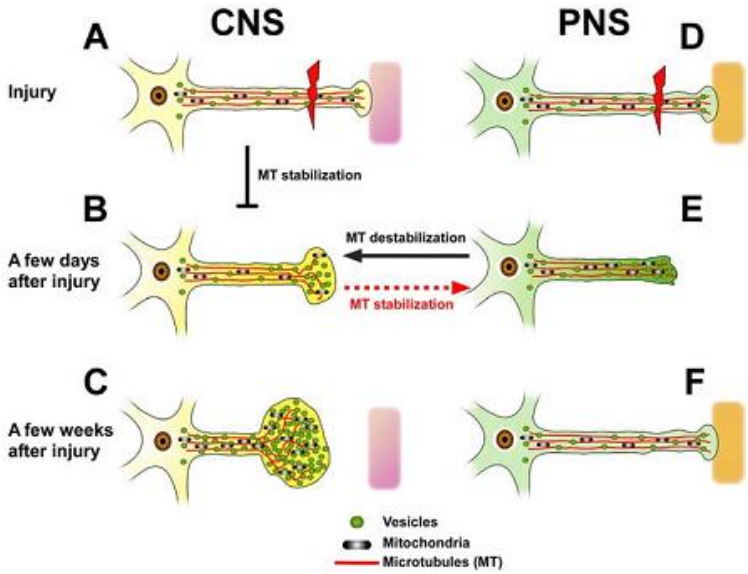


Figure 6: Model for retraction bulb formation versus growth cone-mediated regeneration. A, D, Before injury, PNS and CNS axons are connected to their targets and are functional. B, A few days after injury, the CNS axon proximal to axotomy responds to injury by forming a retraction bulb, which is characterized by dispersed microtubules and accumulated organelles. Stabilization of the microtubules starting immediately after CNS injury, inhibits the formation of retraction bulbs and may induce axon outgrowth. E, The PNS axon forms a growth cone after a lesion which allows for axon elongation. Destabilization of microtubules at this stage can convert the growth cones into retraction bulb-like structures. C, A few weeks after injury, the retraction bulb still enlarges with the accumulation of organelles. F, In contrast, the PNS axon continues its elongation until connecting to the target. Taken from [44].

4. Glycogen synthase kinase 3β (GSK3β) as a putative candidate in axonal regeneration

GSK3 proteins are serine/threonine kinases that were originally identified as key regulatory enzymes in glucose metabolism, phosphorylating glycogen synthase downstream of insulin signaling. There are two mammalian GSK3 isoforms, GSK3α and β, showing high sequence homology with each other across species; 85% total and 95% kinase domains homology [45, 46]. In rodents and humans an alternative splice variant of GSK3β – GSK3β2 has been reported, specifically expressed in the nervous system [46]. Although the name suggests functions restricted to glycogen synthesis, recently GSK3 has emerged as a fundamental regulatory kinase in the nervous system, ranging from neural development to mood stabilization and neurodegeneration [47]. Actually, changes in GSK3 activity have been associated with many psychiatric and neurodegenerative diseases such as Alzheimer's disease, schizophrenia and autism spectrum disorders. Importantly, it has become more evident that GSK3 might be a common therapeutic target for different classes of psychiatric drugs [48]. Understanding how GSK3 is regulated could shed light on related signaling pathways and possible therapeutic applications of this kinase.

GSK3 has been demonstrated as a key downstream regulator in signaling pathways engaged by many extracellular cues including Wnts, NGF, epidermal growth factor (EGF) and hedgehog [45]. Unlike many other kinases, GSK3β is usually active in resting cells [47] being activated by phosphorylation of the Y216 residue and inactivated through S9 phosphorylation [49]. Upon activation of the phosphoinositide 3-kinase (PI3K) signaling pathway by neurotrophins, GSK3β is inactivated by Akt/protein kinase B (PKB) through phosphorylation of the S9 residue in its amino-terminal region. Factors that inhibit axon growth, such as semaphorin 3A, activate GSK3β by inhibiting S9 phosphorylation. Other special feature of GSK3β is that it phosphorylates substrates that have been 'primed' by other kinases [47]. Many GSK3 substrates are microtubule-associated proteins (MAPs) including APC, collapsin response mediator protein 2 (CRMP2), MAP1b, microtubule-associated protein (MAP2), tau, neurofilament and kinesin light chain [45]. Both APC and

CRMP2 are examples of 'primed' substrates. The ability of CRMP2 to bind to tubulin dimers is abolished when it is phosphorylated by GSK3β [50]. Similarly, phosphorylation of APC by GSK3β prevents its ability to bind to microtubules. Thus, inactivation of GSK3β results in CRMP2 and APC dephosphorylation, which leads to microtubule polymerization and stabilization, respectively. On the other hand, MAB1b is thought to be an unprimed substrate [47]. GSK3β is activated by neurotrophins via the ERK pathway and, phosphorylation of MAP1b by GSK3β positively regulates actions of the first, namely ensuring microtubule dynamics [45, 47]. Inhibition of GSK3β activity abolishes MAP1b phosphorylation and reduces microtubule dynamics [47]. Remarkably, it has been shown that phosphorylated MAP1b co-localizes with dephosphorylated APC and CRPM2 at the distal ends of growing axons [47, 51]. There is emerging evidence that S9 phosphorylation has little effect on GSK3β activity towards substrates that do not require 'priming' [52]. Thus, GSK3β may phosphorylate MAP1b and maintain microtubule dynamics even when it is phosphorylated at S9 [47]. This implies that GSK3β can simultaneously activate some substrates and inactivate others, which indicates that when phosphorylated on S9, GSK3 is only partially inactivated. This mechanism provides coordinated regulation of microtubule polymerization, stabilization and dynamics which ensures optimal microtubule assembly in axons. It is presumed as well that GSK3β almost certainly regulates actin filaments [47].

Studies in hippocampal neurons show that the inactive form of GSK3β is located at the tip of each neurite before polarization, but when neurons begin to polarize and one of these neurites develops into the axon, pGSK3β becomes concentrated at the axonal tip. This suggests that local inactivation of GSK3β at the nascent axon is fundamental for polarization [50]. However, recently a mutant mice (GSK3αS21A/GSK3βS9A double knockin (KI) mice) did not show overt phenotype in the nervous system, in particularly they developed normal neuronal polarity [53]. This suggests that alternative pathways might regulate GSK3 activity. Studies so far have concerned the inhibitory mechanism of GSK3. Nevertheless, it is becoming increasingly apparent that activation of GSK3 is an important aspect [46]. In fact, besides the crucial role attributed to S9 phosphorylation, Y216 might have an essential role on GSK3β regulation (Figure 7). Tyrosine

phosphorylation is thought to occur by signal-dependent regulation or through autophosphorylation, facilitating GSK3 activity by promoting substrate accessibility [46]. As a matter of fact, in mammalian cells, the tyrosine kinases Fyn, Pyk2 and Csk have been implicated in GSK3 phosphorylation. Pyk2 is thought to mediate lysophosphatidic acid (LPA)-induced activation of GSK3 by phosphorylation on Y216 residue, during neurite retraction [54]. On the other hand, Cohen and coworkers clearly show that Y216 phosphorylation is an autophosphorylation event [55].

It has been observed that strong inhibition of GSK3 activity dramatically reduces axon growth in immature and adult DRG neurons. In contrast, incomplete GSK3 inactivation increased axon length[3]. It seems that GSK3 activation contributes to axon growth repression, but a degree of GSK3 activity is necessary for efficient axon extension because of the dual roles of GSK3 in activating and inactivating different substrates during microtubule assembly [45]. Recently, Dill and his collaborators claimed that inactivation of GSK3β stimulates significant neurite elongation and axonal growth *in vitro* and promote axonal regeneration and functional recovery *in vivo* after traumatic CNS axonal injuries [3]. Nevertheless, a lot of debate is still going on which role GSK3β performs in axonal regeneration, as some reports describe inactivation of GSK3β as causing neurite outgrowth inhibition in cerebellar and DRG neurons, mimicking the inhibitory effects of myelin [56]. Actually, Alabed and coworkers propose a model in which GSK3 myelin-dependent inactivation by S9 phosphorylation, causes collapsin response-mediated protein 4 (CRMP4) dephosphorylation and consequently enhancing L-CRMP4-RhoA interaction, that mediates the inhibitory effects.

Most of the members of GSK3β pathway were recently detected in our laboratory, with a fold change CL/SCI compatible with a crucial role of GSK3β in the gain of regenerative capacity following conditioning lesion (CL). This identification was performed recurring to proteomic screening based on phosphoproteomics by Kinexus arrays, which enables to identify and quantify phosphorylating proteins in key signaling pathways, and iTRAQ (isobaric tag for relative and absolute quantitation), that enables the identification and quantification of proteins from different sources in a single experiment, following a non-gel based approach.

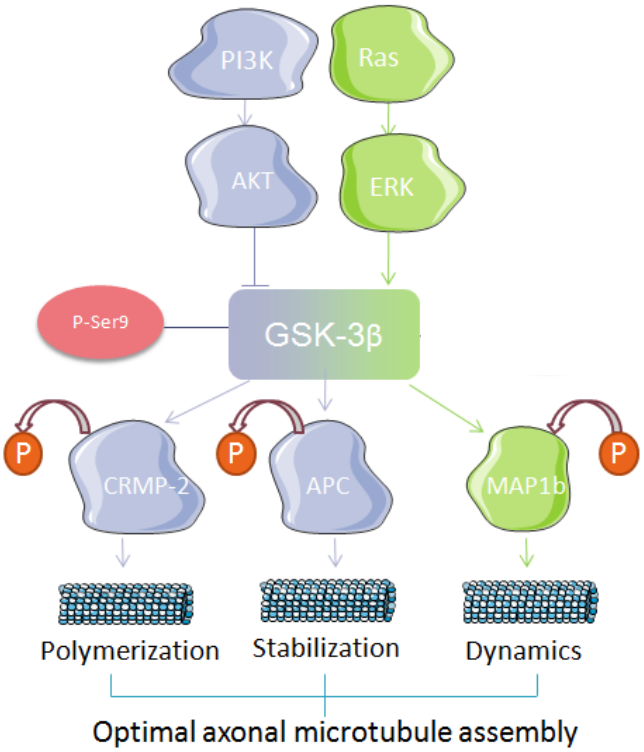


Figure 7: Representation of the three aspects of microtubule assembly in axons, regulated by GSK3β: microtubule polymerization, stabilization and dynamics. In the PI3K pathway, Akt inactivates GSK3β by S9 phosphorylation, which in turn dephosphorylates CRMP2 and APC. This promotes microtubule polymerization and stabilization. In the Ras pathway, ERK phosphorylates and activates GSK3β which will phosphorylate MAP1b leading to microtubule dynamics enhancement. These three aspects act together to establish optimal axonal microtubule assembly.

Objectives

The main objective of this work was to understand how GSK3β is regulated in order to ultimately promote axonal regeneration. To achieve this goal, the following strategies were pursued:

1. Validate the modulation of the GSK3β pathway in the conditioning lesion (CL) model
2. Evaluate the role of GSK3β Y216 phosphorylation in axonal regeneration
3. Identify the mechanism responsible for the modulation of Y216 phosphorylation
4. Determine the role of GSK3β regulation *in vivo* using GSK3βS9A KI mice

Materials and Methods

Surgical procedures

Rats were handled according to European Union and National rules. All animals were maintained under a 12h light/dark cycle and fed with regular rodent's chow and tap water *ad libitum*. In order to perform the lesions described below, Wistar rats with ≈8-10 weeks old were used.

Sciatic nerve injury: animals were anesthesized with a mixture of ketamine and medetomidine (0,5mg/kg of medetomidine and 75mg/kg of ketamine). The skin was shaved, the sciatic nerve was exposed and a transection at the level of the mid-thigh was performed with a scissor. Analgesia was performed 48h following injury, with buthorphenol, 1mg/kg twice a day.

Spinal cord hemisection: animals were anesthesized with ketamine/medetomidine as mentioned above. Spinal cord hemisection at the dorsal thoracic (T7) level was performed with a microscissor. The postoperative treatment consisted of subcutaneous injection of buthorphenol 1mg/kg twice a day for 72h (analgesia); daily subcutaneous injection of 5ml of Duphalyte for 72h (fluid therapy) and manual voiding of the bladder twice a day during the time of the experiment. Wet food was placed in the cage floor and water with antibiotic (0,016% baytril) was supplied in long nipple bottles.

Conditioning lesion animals: animals were subjected to sciatic nerve transection followed by spinal cord hemisection approximately 1 week later.

Primary cultures of DRG neurons

DRGs were aseptically dissected, freed of roots and treated with 0,125% collagenase (Sigma) for 90 min at 37°C with 5%CO_2. After enzyme treatment, a single-cell suspension

was obtained by trituration with a fire-polished Pasteur pipette. The cell suspension was centrifuged into a 15% albumin gradient for 10 min at 10000g. The resulting pellet was resuspended in DMEM/F12 medium (Sigma) supplemented with B27 (Invitrogen) and 50 ng/ml of nerve growth factor (Milipore), plated in poly-L-lysine/laminin coated 13mm coverslips and maintained at 37°C with 5%CO_2.

Neurite outgrowth of DRG neuron cultures

DRG neuron cultures were performed as described previously. To determine the neurite outgrowth of DRG neurons in the presence of LPA (Sigma), lumbar (L4-L6) DRG neurons from animals with sciatic nerve injury were cultured for 8h at 37°C. LPA (100µM) was added 1h before ending the cultures. In order to evaluate the effect of GSK3 inhibitors, neurite outgrowth of uninjured DRG neurons cultured for 15h in the presence of different inhibitors was performed. Inhibitor α-4-dibromoacetophenone or inhibitor VII (1µM and 10µM, Calbiochem), inhibitor 6-bromoindirubin-3'-oxime or inhibitor X (30nM and 300nM, Calbiochem) and inhibitor SB415286 (7,5µM and 50µM, Sigma) were used.
After fixation with 4% paraformaldehyde (PFA), cells were immunostained with βIII-tubulin. Briefly, cells were permeabilized with Triton-X 100, blocked in blocking buffer (5% fetal bovine serum, 0,4% Tween-20 in PBS) for 1h at room temperature and incubated with the primary antibody anti βIII-tubulin (1:2000, Promega) in blocking buffer overnight at 4°C. The cells were later incubated with Alexa488 anti-mouse IgG (1:1000, Invitrogen) in blocking buffer for 1h at room temperature and finally mounted with Vectashield with DAPI (Vector laboratories). Neurite outgrowth measurement was carried out from 20x magnified fields using Neuron J plugin for ImageJ, a public domain JAVA image-processing program. At least 100 cells were analysed for each condition.

Neurite outgrowth of DRG neuron cultures from GSK3βS9A knockin mice

GSK3βS9A knockin mice were kindly provided by Dario R. Alessi (MRC Protein Phosphorylation Unit, Dundee, Scotland) [57]. DRG neuron cultures were performed as

described previously and grown for 14 hours (uninjured neurons, Un) or kept in culture for 24 hours, treated with trypsin and grown for 14 hours (conditioned neurons, cDRG). Cells were then fixed for neurite outgrowth measurement, performed as above mentioned.

Gene transfection

The following constructs were used: GFP-tagged versions of kinase-dead Pyk2 (Pyk2 K457A) were provided by Drs. Sancho and Sanchez-Madrid (Hospital de la Princesa, Madrid, Spain). GSK3βWT plasmid was kindly provided by Dr. Michal Hetman [58]. The GSK3βWT DNA fragment was excised from the plasmid with SmaI and XbaI and inserted into pEGFP-C1 (Clontech), which was used to produce the GSK3βY216F mutant (using the Quickchange kit; Stratagene). Mutants were constructed using two mismatched primers (5'-GGAGAGCCCAATGTTTCATTTATCTGTTCTCGGTACTAC-3' and 5'-GTAGTACCGAGAACAGATAAATGAAACATTGGGCTCTCC-3'). Minipreps of plasmid DNA were tested by sequencing at SEQLAB (Germany).

DRG neurons from animals with SCI were transfected with the DNA constructs (EGFP; GSK3βY216F and Pyk2KD) according to the Amaxa protocol for mouse neurons. Briefly, dissociated neurons were spun down to remove the supernatant completely and resuspended in 100µl specified Amaxa electroporation buffer with 4µg plasmid DNA. Suspended cells were then transferred to a 2,0mm cuvette and electroporated with an Amaxa Nucleofector apparatus. After electroporation, cells were cultured in suspension in uncoated plastic wells for 24h and then transferred to poly-L-lysine/laminin coated 13mm coverslips and kept in culture for 16 hours. Cells were then fixed for neurite outgrowth measurement as previously described.

Western blotting

DRGs (L4-L6) from animals either uninjured, with SCI alone or conditioning lesion (CL) were collected. Samples of spinal cord (T7-T8) of uninjured animals or animals with SCI or

conditioning lesion (CL) were dissected. Protein lysates were prepared by homogenizing the tissues in Kinexus buffer (20mM MOPS, 2mM EGTA, 5mM EDTA, 30mM NaF, 60mM β-glycerophosphate, 20mM sodium pyrophosphate, 1mM sodium orthovanadate, 1% Triton X-100, 1% DTT), 1mM PMSF and protease inhibitor cocktail (GE Healthcare). For cell lysates of DRG neurons, cultures were performed as described above and cells were lysed in Kinexus buffer with a cell scraper. After sonication, samples were centrifuged at 15000gmax for 10min at 4°C. Total protein quantification was assessed using the Bio-Rad DC protein assay reagents. For Western blot of conditioned DRG cultures in the presence of LPA, DRG neurons were treated with LPA for 0, 5, 30 and 60 minutes.

Protein lysates (25-50µg/lane) were run on 10% or 12% SDS-PAGE gel and then transferred to a nitrocellulose membrane (Amersham). For the anti-phospho-MAP1b and anti-MAP1b identification, Criterion XT Precast Gel 3-8% Tris-Acetate (Bio-Rad) was used. For Western analysis, membranes were blocked with blocking buffer (5% nonfat dried milk in TBS 0,1% Tween 20) and incubated overnight at 4°C in 5% BSA in TBS 0,1% Tween 20 with primary antibodies. In the case of the CRMP2 Western blot, the membrane was incubated for 1h room temperature in 5% milk in TBS 0,1% Tween 20 with primary antibodies. The following primary antibodies were used: rabbit anti-phospho-Akt(S473) (1:2000, Cell Signaling), rabbit anti-Akt (1:1000, Cell Signaling), rabbit anti-phospho-GSK3β(S9) (1:1000, Cell Signaling), rabbit anti-phospho-GSK3β(Y216) (1:2000, Santa Cruz Biotechnology), mouse anti-GSK3α/β (1:3000, Santa Cruz Biotechnology), rabbit anti-phospho-Src(Y529) (1:500, Signalway antibody), rabbit anti-Fyn (1:1000, Cell Signaling), goat anti-Pyk2 (1:1000, Santa Cruz Biotechnology), sheep anti-phospho-CRMP2(T509/514) (1:1000, Kinasource), sheep anti-CRMP2 (1:500, Kinasource), mouse anti-phospho-MAP1b(T1265) (1:500, Novus Biologicals), mouse total MAP1b (1:2500, kindly provided by Dr Itzhak Fischer, Drexel University College of Medicine) and mouse anti-GAPDH (1:2000, Santa Cruz Biotechnology). After washing, membranes were incubated with secondary antibodies for 1h at room temperature. The antibodies used were anti-rabbit HRP (1:10000, Jackson Immunoresearch), anti-mouse HRP (1:10000, Thermo Scientific) and anti-goat/sheep (1:20000, Binding Site). Proteins were detected using a chemiluminescent substrate, Pierce ECL Western blotting substrate (Thermo

Scientific). For each experiment representative Western blots are shown. Densitometry was performed with QuantityOne software (Bio-Rad).

Results

5. Validation of GSK3β pathway in the conditioning lesion model

Our group has previously performed a screening to search for protein candidates that could be responsible for the gain of CNS regeneration capacity in the conditioning lesion model. Two proteomic approaches, iTRAQ and Kinexus arrays (Kinetworks phospho-site broad coverage pathway screen) were used in order to identify altered proteins and phosphorylated forms of key signaling proteins in extracts of DRG from adult rats with conditioning lesion (CL) or spinal cord injury (SCI). Interestingly, several proteins belonging to GSK3β pathway were modified when comparing both groups, as can be concluded from the CL/SCI ratio represented in Table 1. However, these results required validation by additional techniques such as Western blotting.

Table 1: List of proteins from the GSK3β pathway differentially regulated after conditioning lesion (CL) as determined by iTRAQ and Kinexus proteomics.

	CL/SCI
Protein kinase B alpha (Akt) [S473]	Kinexus: 1,53
Glycogen synthase kinase 3 beta (GSK3β) [S9]	Kinexus: 1,55
Glycogen synthase kinase 3 beta (GSK3β) [Y216]	Kinexus: 0,53
Collapsin response mediator factor 2 (CRMP2)	iTRAQ: 0,83
Microtubule associated protein 1b (MAP1b)	iTRAQ: 0,80

5.1. GSK3β pathway is differentially regulated by the conditioning lesion in the DRG

The first part of this project consisted on the validation of the GSK3β pathway in the conditioning lesion model given the differences observed in the DRG in the above mentioned proteomic approaches and considering the putative importance of GSK3β in the gain of regenerative capacity following conditioning lesion. For that, we performed Western blotting in the DRG, for proteins belonging to the GSK3β pathway differentially regulated according to iTRAQ and Kinexus. In addition to samples from animals with SCI or conditioning lesion, we analysed samples from uninjured animals to determine the protein alterations caused by the lesion. The levels of phosphorylated Akt (pAKT(S473); the kinase responsible for GSK3β(S9) phosphorylation) were similar between uninjured (Un) and SCI animals but the animals with conditioning lesion (CL) exhibited an increase in pAkt(S473) (Figure 8) which is in agreement with the Kinexus results. In what concerns to pGSK3β(S9) (the inactive form of GSK3β), we determined an increase in the conditioning lesion (CL) model, when compared to the SCI model and to the uninjured (Un) animals (Figure 9). This is in agreement with the differences observed for pAkt and also with the result obtained by Kinexus analysis. Concerning pGSK3β(Y216) (the active form of GSK3β), the phosphorylation of this protein in this particular residue is diminished in the conditioning lesion (CL), comparing to the SCI and to uninjured (Un) animals (Figure 10) which validates the result obtained by Kinexus. In respect to the GSK3β substrate CRMP2, we only assessed animals with SCI or conditioning lesion (CL). It has been described that CRMP2 contains two isoforms, CRMP2A (75kDa) and CRMP2B (64kDa) which share a common targeted epitope at the C-terminal end [59]. In the DRG, we were only able to quantify the CRMP2B isoform which showed a decrease after conditioning lesion (CL) when compared to the SCI model (Figure 11). The proteomic approach iTRAQ did not distinguish the two isoforms but showed a decrease of total CRMP2 after conditioning lesion (CL) in comparison with SCI only. These results validated the proteomic techniques used previously and suggest that after conditioning lesion, GSK3β activity diminishes in the DRG.

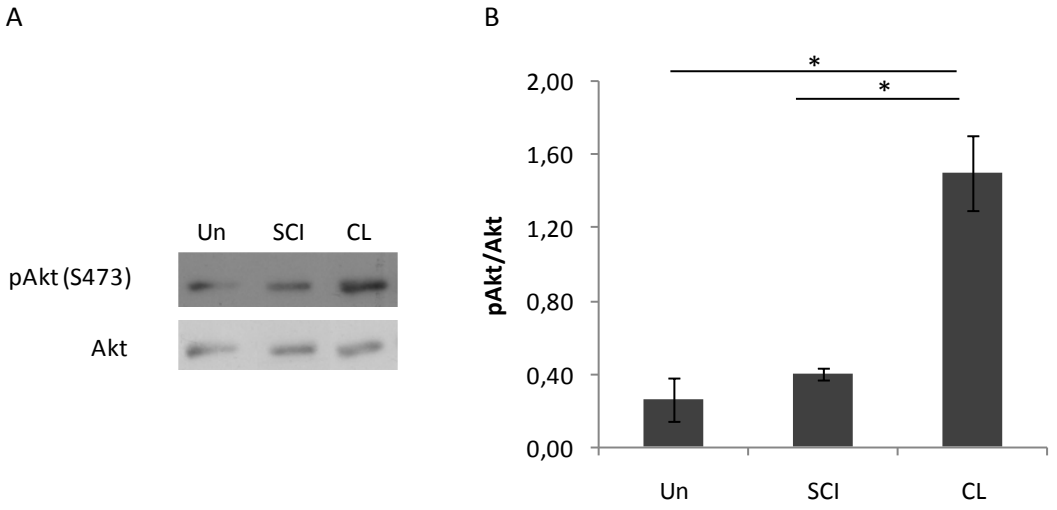


Figure 8: Akt(S473) is increasingly phosphorylated in the conditioning lesion model

Western blot analysis (A) and respective quantification (B) of the relative amount of pAkt (S473) in DRG extracts of uninjured (Un), spinal cord injury (SCI) and conditioning lesion (CL) animals. Results are presented as mean±sem. Two-tailed Student's t test with $p<0,05$ (*).

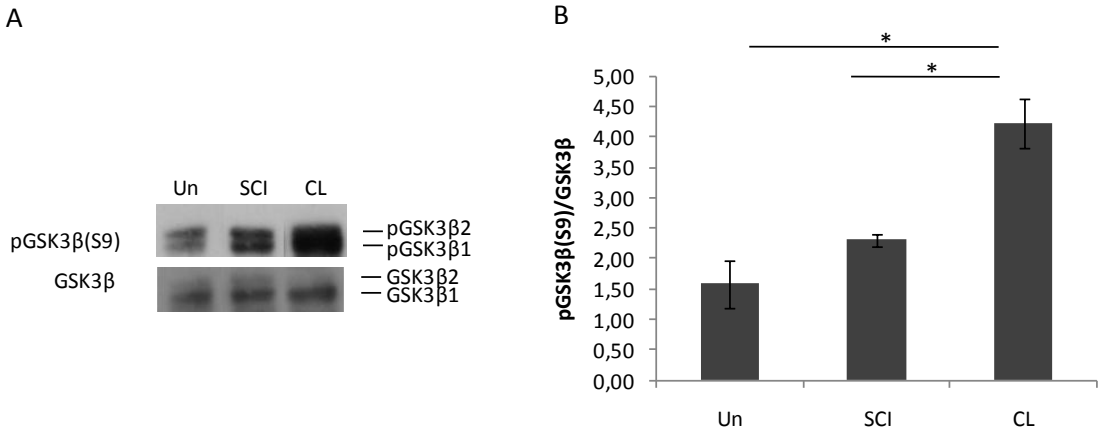


Figure 9: GSK3β(S9) phosphorylation is increased after conditioning lesion

Western blot analysis (A) and respective quantification (B) of the relative amount of pGSK3β(S9) in DRG extracts of uninjured (Un), spinal cord injury (SCI) and conditioning lesion (CL) animals. Results are presented as mean±sem. Two-tailed Student's t test with $p<0,05$ (*).

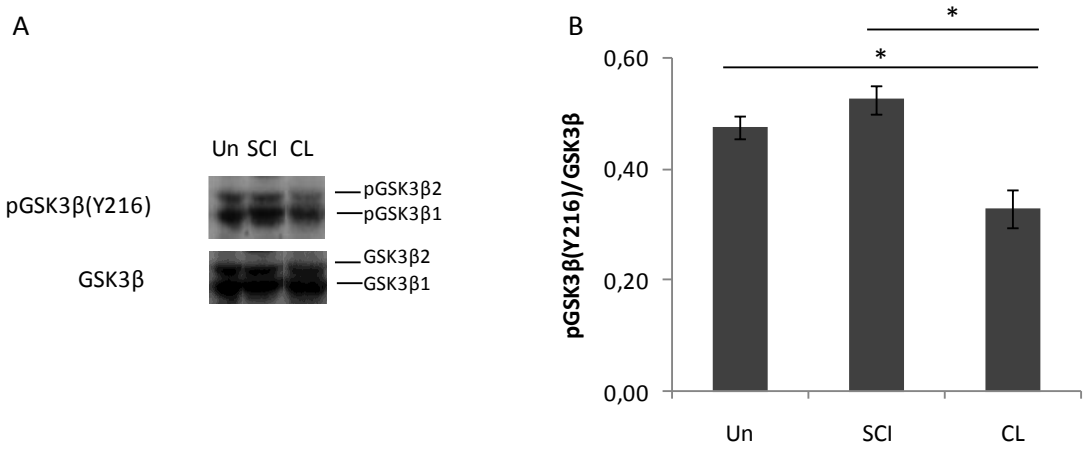


Figure 10: GSK3β(Y216) phosphorylation is downregulated in the conditioning lesion model

Western blot analysis (A) and respective quantification (B) of the relative amount of pGSK3β(Y216) in DRG extracts of uninjured (Un), spinal cord injury (SCI) and conditioning lesion (CL) animals. Results are presented as mean±sem. Two-tailed Student's t test with $p<0,05$ (*).

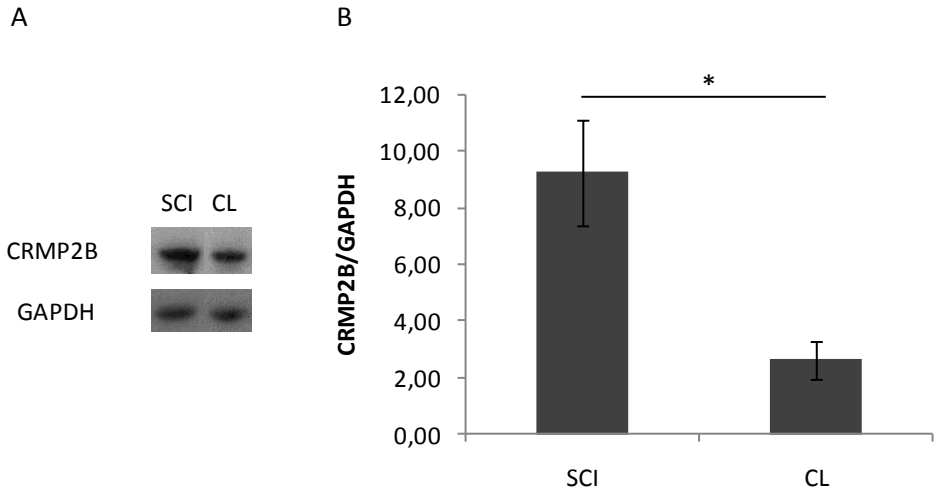


Figure 11: CRMP2B total levels are decreased after conditioning lesion

Western blot analysis (A) and quantification (B) of the relative amount of CRMP2B in DRG extracts of spinal cord injury (SCI) and conditioning lesion (CL) animals. Results are presented as mean±sem. Two-tailed Student's t test with $p<0,05$ (*).

5.2. The conditioning lesion leads to a decrease in GSK3β phosphorylation at Y216 in the spinal cord injury site

The GSK3β pathway plays an important role in axonal regeneration by local regulation of the cytoskeletal dynamics of the growth cone. As such we evaluated GSK3β regulation in the spinal cord injury site. We observed that after injury there is a decrease of pGSK3β(S9) in the lesion site which is not altered by a conditioning lesion (CL) (Figure 12). In the case of Y216 phosphorylation, the levels of pGSK3β(Y216) are decreased after SCI. Compared with levels in animals with SCI, animals with conditioning lesion present decreased levels of pGSK3β(Y216) in the lesion site (Figure 13). Although the majority of research on GSK3β has focused on the inhibitory phosphorylation at S9, our data shows a decrease in the levels of pGSK3β(Y216) from animals with conditioning lesion when compared to animals with SCI. Moreover, no difference was observed in the amount of pGSK3β(S9) between these models. Together our results suggest that downregulation of GSK3β(Y216) phosphorylation in the lesion site might underlie the gain of regenerative capacity in the conditioning lesion model. In respect to the GSK3β substrates MAP1b and CRMP2, we only assessed animals with SCI or conditioning lesion (CL). Interestingly, we did not identify any phosphorylated MAP1b(T1265) after SCI, contrarily to the increase observed in the conditioning lesion (CL) in the injury site (Figure 14). Regarding CRMP2, as mentioned above two isoforms exist, but we were only able to quantify CRMP2A in the injury site. Our results show that in the spinal cord after conditioning lesion (CL), there is a decrease of the phosphorylated form of CRMP2A(T509/514) when compared to SCI (Figure 15). These results show that the decrease in GSK3β kinase activity due to downregulation of Y216 phosphorylation in the conditioning lesion (CL) model leads to a decrease in phosphorylation of CRMP2 which is required for axonal elongation. In the case of MAP1b, we only observed the phosphorylated form of the protein in the conditioning lesion (CL) model which is not in accordance with a decrease in GSK3β activity, but is in agreement with the fact that phosphorylated MAP1b is required for axonal growth.

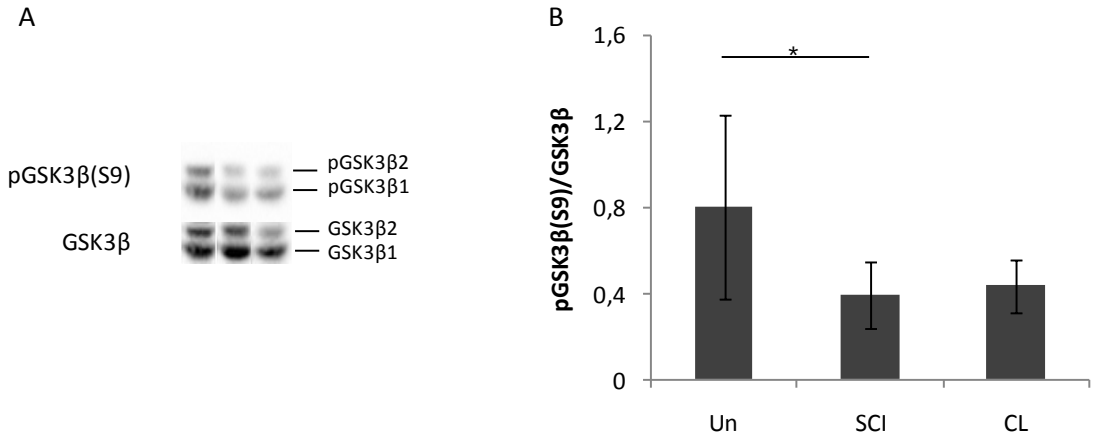


Figure 12: GSK3β(S9) phosphorylation is increased after conditioning lesion

Western blot analysis (A) and respective quantification (B) of the relative amount of pGSK3(S9) in spinal cord extracts of uninjured (Un), spinal cord injury (SCI) and conditioning lesion (CL) animals. Results are presented as mean±sem. Two-tailed Student's t test with $p<0,05$ (*).

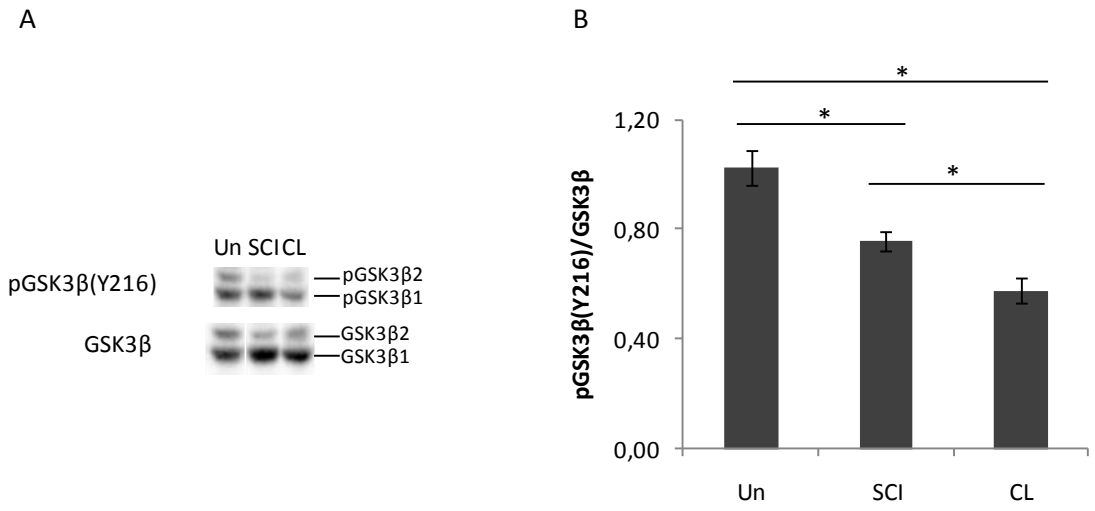


Figure 13: GSK3β(Y216) phosphorylation is downregulated in the conditioning lesion model

Western blot analysis (A) and quantification (B) of the relative amount of pGSK3β(Y216) in spinal cord extracts of uninjured (Un), spinal cord injury (SCI) and conditioning lesion (CL) animals. Results are presented as mean±sem. Two-tailed Student's t test with $p<0,05$ (*).

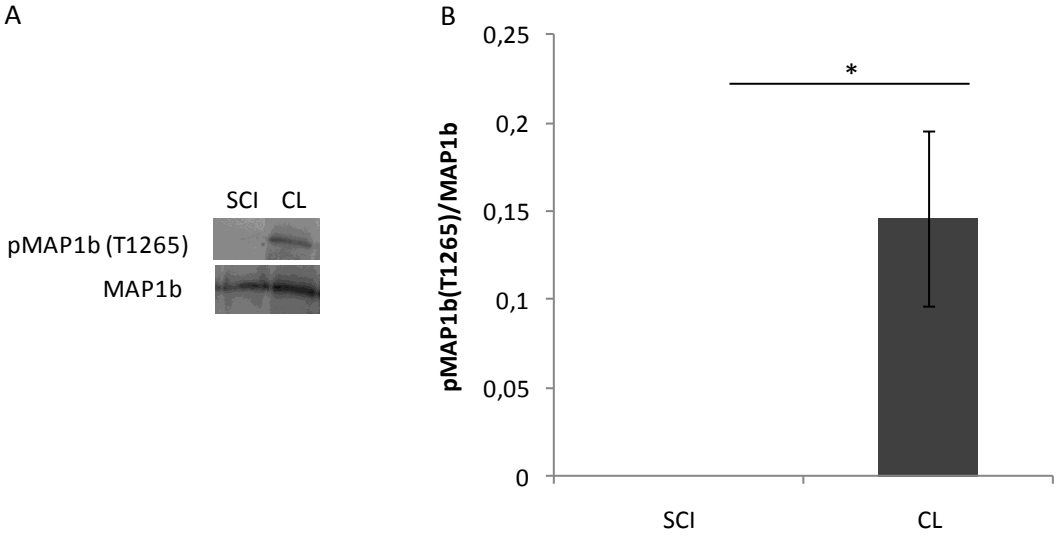


Figure 14: MAP1b(T1265) phosphorylation is upregulated in the conditioning lesion

Western blot analysis (A) and quantification (B) of the relative amount of pMAP1b(T1265) in spinal cord extracts of spinal cord injury (SCI) and conditioning lesion (CL) animals. Results are presented as mean±sem. Two-tailed Student's t test with $p<0,05$ (*).

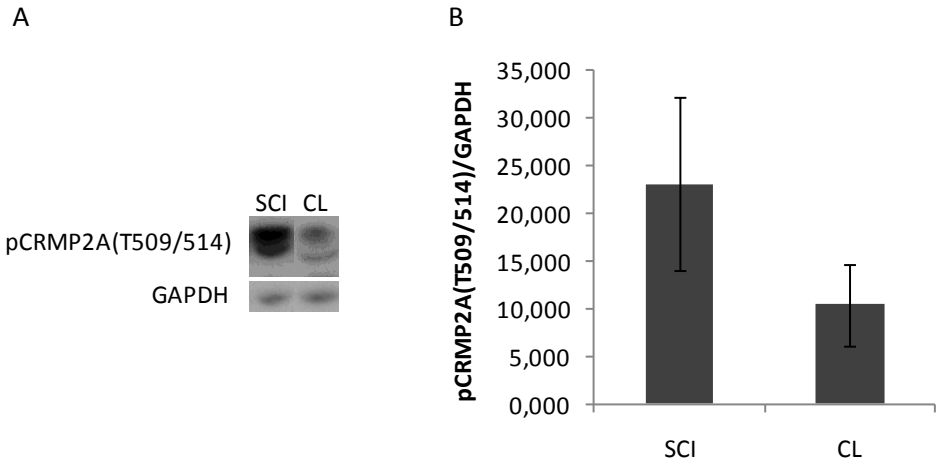


Figure 15: CRMP2A(T509/514) phosphorylation is decreased after conditioning lesion

Western blot analysis (A) and quantification (B) of the relative amount of pCRMP2(T509/514) in spinal cord extracts of spinal cord injury (SCI) and conditioning lesion (CL) animals. Results are presented as mean±sem. Two-tailed Student's t test with $p<0,05$ (*).

6. Evaluation of the role of GSK3β(Y216) phosphorylation in axonal regeneration

6.1. *Induction of Y216 phosphorylation decreases the conditioning effect in vitro*

The second part of this project intended to evaluate the role of GSK3β(Y216) phosphorylation in the promotion of axonal regeneration. We addressed this question by performing DRG cultures of conditioned DRG neurons (animals with SNI) in the presence of LPA, a compound that increases Y216 phosphorylation of GSK3β. With this approach we could answer the question whether a decreased phosphorylation of GSK3β at Y216 is required for the conditioning effect.

Initially, we evaluated the phosphorylation status of Y216, by performing Western blotting of lysates from conditioned DRG cultures in the presence of LPA for the following time points: 0, 5, 30 and 60 minutes, unraveling that at 30 minutes, the phosphorylation of GSK3β(Y216) is maximal (Figure 16). Then, we performed neurite outgrowth to unravel the role of GSK3β(Y216) phosphorylation in the promotion of axonal regeneration by the conditioning effect. Representative pictures for uninjured (Un), conditioned DRG (cDRG) and conditioned DRG in the presence of LPA (cDRG+LPA) are presented in Figure 17. In agreement with the literature, the conditioning effect was observed, as determined by the increase in percentage of cells with neurites, mean length and length of the longest neurite for conditioned DRG (cDRG) in comparison with the uninjured (Un). Moreover, the branching (number of segments) decreases for conditioned DRG (cDRG) when compared to uninjured (Un). The LPA treatment decreased the percentage of cells with neurites and the mean length when compared to conditioned DRG (cDRG) (Figure 18). In order to elucidate better what happens in terms of axonal extension in our conditions, the length of the longest neurite was scored according to particular lengths and the corresponding percentage of cells with neurites was calculated. Again, we can see that the conditioning effect is working, as in the conditioned DRG (cDRG) we observed less smaller neurites (<100μm) and more longer ones (100-350μm and >350μm) than in the

uninjured (Un) animals. Addition of LPA to conditioned DRG (cDRG) caused a rise in the percentage of cells with smaller neurites and a decrease of intermediate and longer neurites (Figure 19).

Our results suggest that a decrease in Y216 phosphorylation of GSK3β might have an important role in the promotion of axonal growth by the conditioning effect.

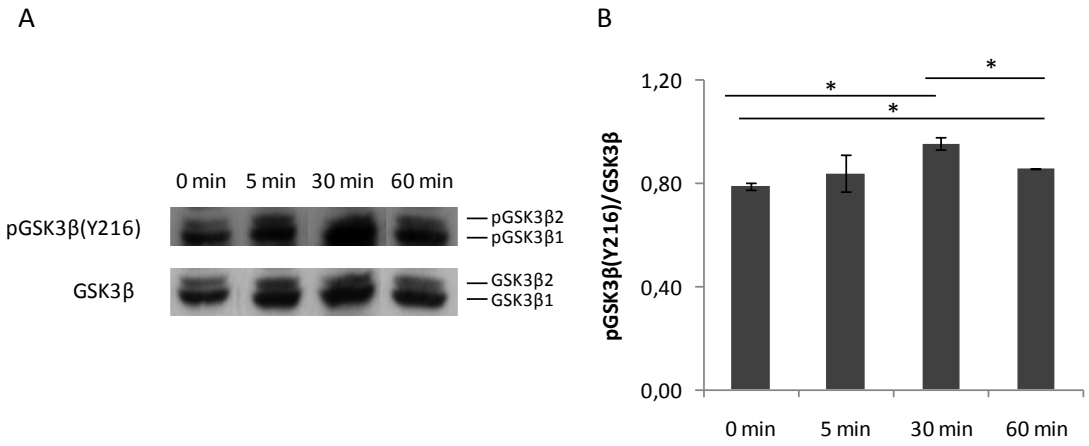


Figure 16: GSK3β(Y216) phosphorylation reaches maximum levels with LPA treatment for 30 min

Western blot analysis (A) and respective quantification (B) of the relative amount of pGSK3β(Y216) present in lysates from conditioned DRG cultures. Results are presented as mean±sem. Two-tailed Student's t test with $p<0,05$ (*).

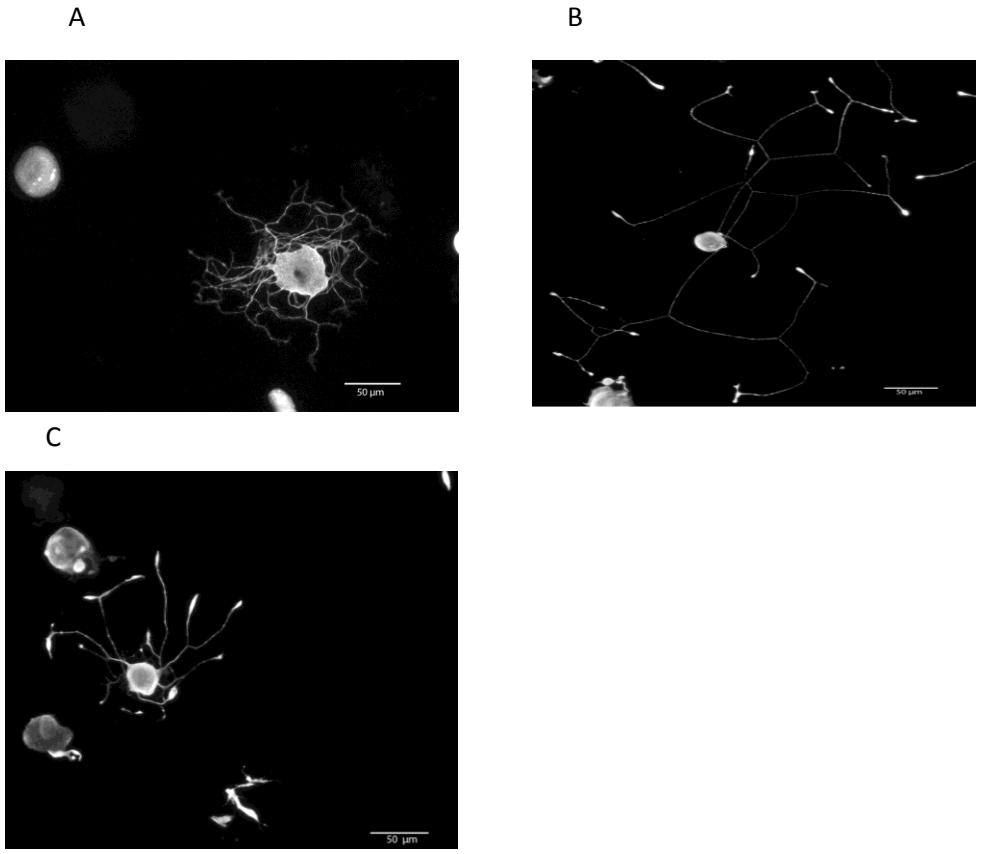


Figure 17: Representative pictures of DRG cultures stained for βIII-tubulin. A. DRG cultures from uninjured animals (Un); B. DRG cultures from animals with SNI (cDRG); C. DRG cultures from animals with SNI, in the presence of 100µM LPA (cDRG+LPA).

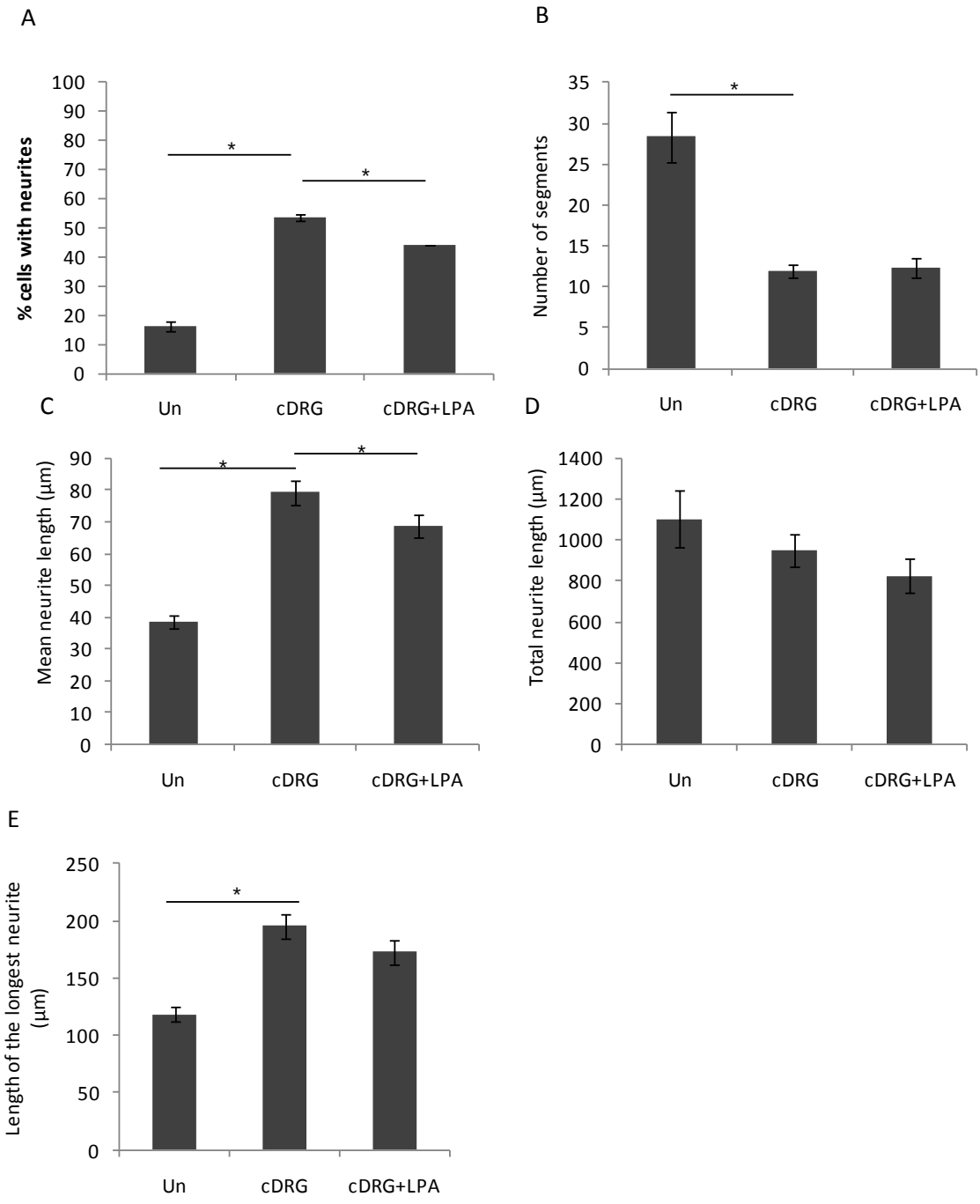


Figure 18: Neurite outgrowth of DRG neurons from uninjured animals (Un) and animals with SNI grown in the absence (cDRG) or presence of 100µM LPA (cDRG+LPA). The results are expressed as mean±sem. Two-tailed Student's t test with $p<0,05$ (*).

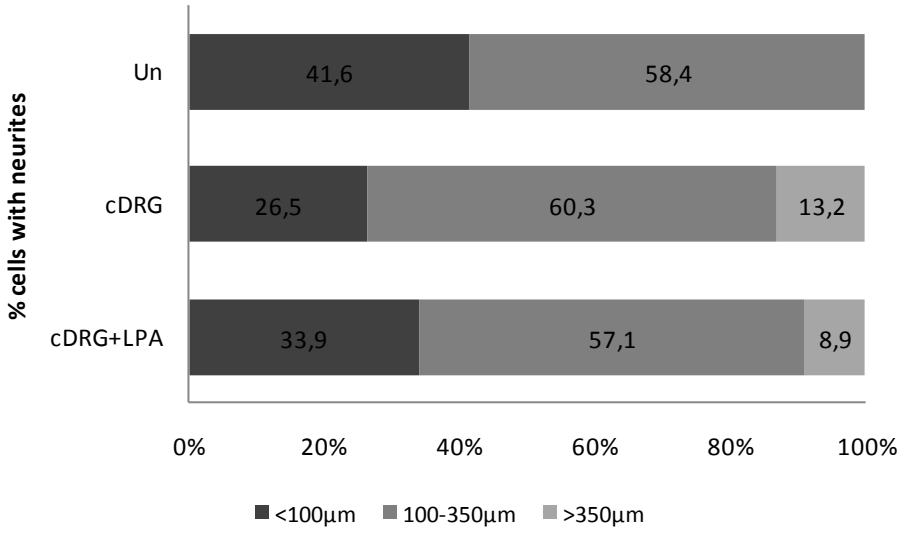


Figure 19: Percentage of cells with neurites for uninjured (Un), conditioned DRG (cDRG) and conditioned DRG in the presence of LPA (cDRG+LPA) which are scored according to: <100µm; 100-350µm or >350µm for the length of the longest neurite.

6.2. Overexpression of GSK3βY216F in DRG neurons isolated from animals with spinal cord injury

To further explore the relevance of GSK3β phosphorylation at Y216 in axonal growth, we analysed the effect of overexpressing GSK3βY216F in DRG neurons from animals with spinal cord injury. We had observed that animals with spinal cord injury present an increase in pGSK3β(Y216) in comparison with animals with conditioning lesion, so we hypothesized that mutation of the Y216 residue would increase the growth capacity of spinal cord injury neurons. DRG neurons were transfected with the control EGFP vector or with GSK3βY216F cloned into the EGFP vector. Representative images for each condition are shown on Figure 20. The percentage of cells with neurites was higher for the cells transfected with the EGFP-GSK3βY216F. Regarding the number of segments, the EGFP-GSK3βY216F construct leads to an increase in the number of segments. When transfected with EGFP-GSK3βY216F, the cells present a decrease in the mean length. Concerning the total length, no statistically differences were observed between the two conditions. For

the length of the longest neurite, there is a decrease of the length of the longest neurite for the EGFP-GSK3βY216F construct (Figure 21). These results showing that overexpression of GSK3βY216F decreases neurite outgrowth suggest that a minimal kinase activity is required for the GSK3β effect in axonal growth.

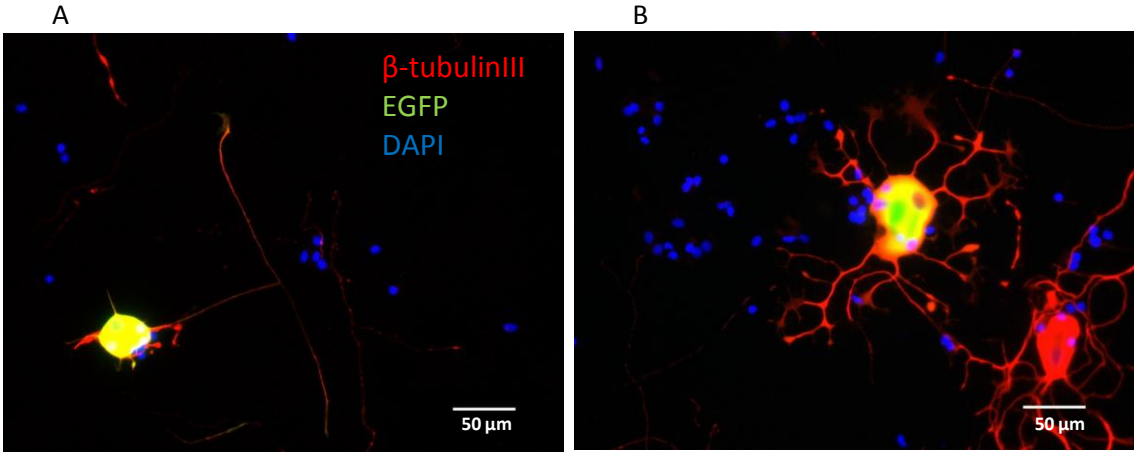


Figure 20: Representative pictures of adult DRG cultures from animals with SCI stained for βIII-tubulin A. cells transfected with control EGFP vector; B. cells transfected with GSK3βY216F cloned into the EGFP vector.

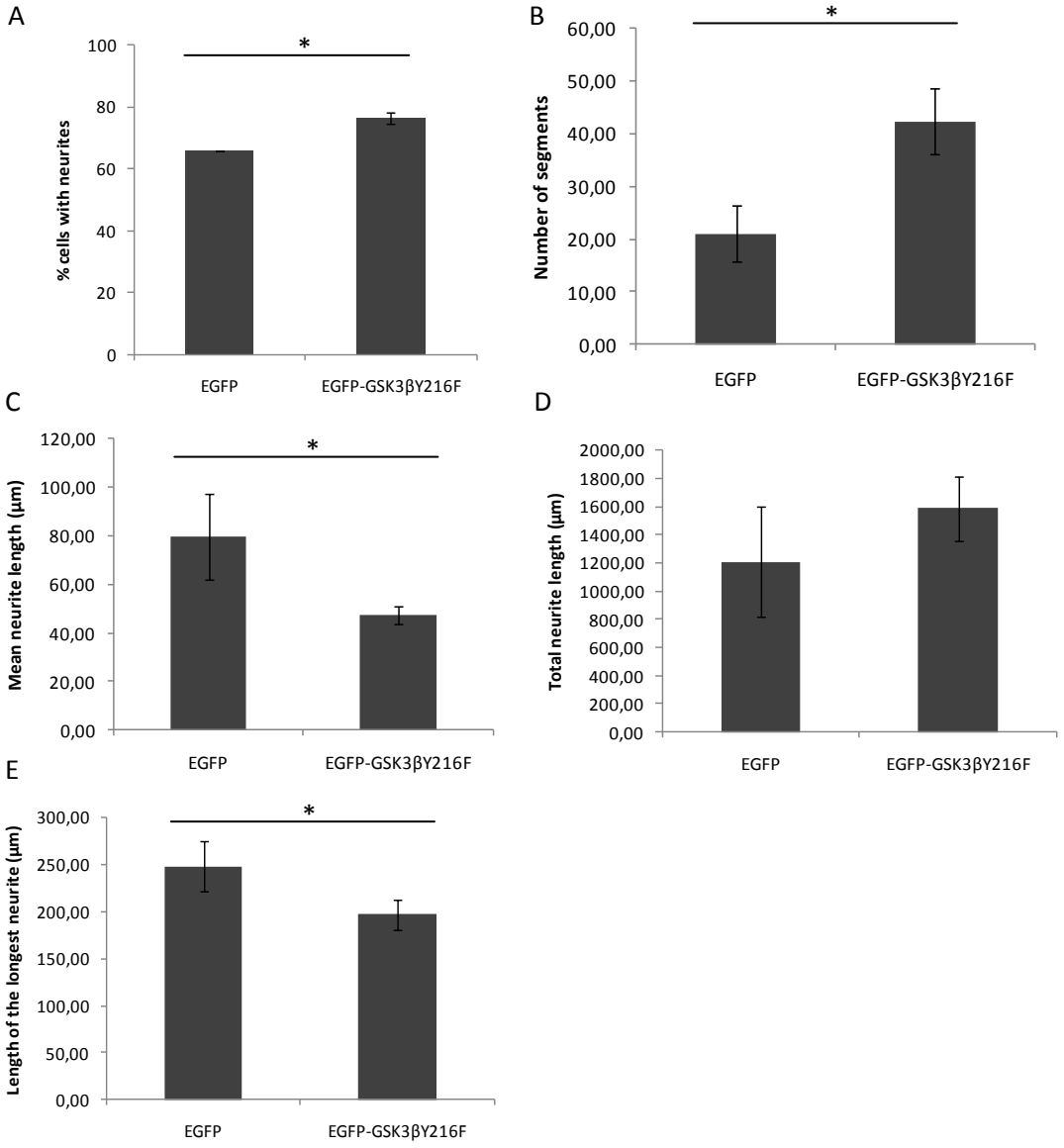


Figure 21: Neurite outgrowth of DRG neurons from animals with SCI transfected with EGFP or EGFP-GSK3βY216F. The results are expressed as mean±sem. Two-tailed Student's t test with $p<0,05$ (*).

6.3. Evaluation of the effect of GSK3β inhibition in the promotion of axonal regeneration by a decrease in Y216 phosphorylation

Our results suggest that the conditioning lesion leads to a decrease in GSK3β activity which promotes axonal regeneration. Based on our observations which demonstrate a regulatory role of pGSK3β(Y216) on axonal growth and taking into account that global

inhibition of GSK3β inhibited axonal regeneration, we decided to analyse the effect of several GSK3β inhibitors on axonal growth and in parallel analyze the phosphorylation status of the protein for the inhibitors having an effect. We treated adult DRG neurons with the following inhibitors: SB415286, 6-bromoindirubin-3'-oxime (inhibitor X) and α-4-dibromoacetophenone (inhibitor VII). Inhibitors were used at a low dose (to check for induction of growth) and at high dose (for inhibition of growth). In Figure 22 are shown representative images of DRG neurons in the presence of inhibitor SB415286. In fact, the only significant differences found for this experiment are related to the percentage of cells with neurites, which increases with the low dose of inhibitor, and to the mean length and length of the longest neurite, which present a decrease for the highest dose in comparison with the control (Figure 23).

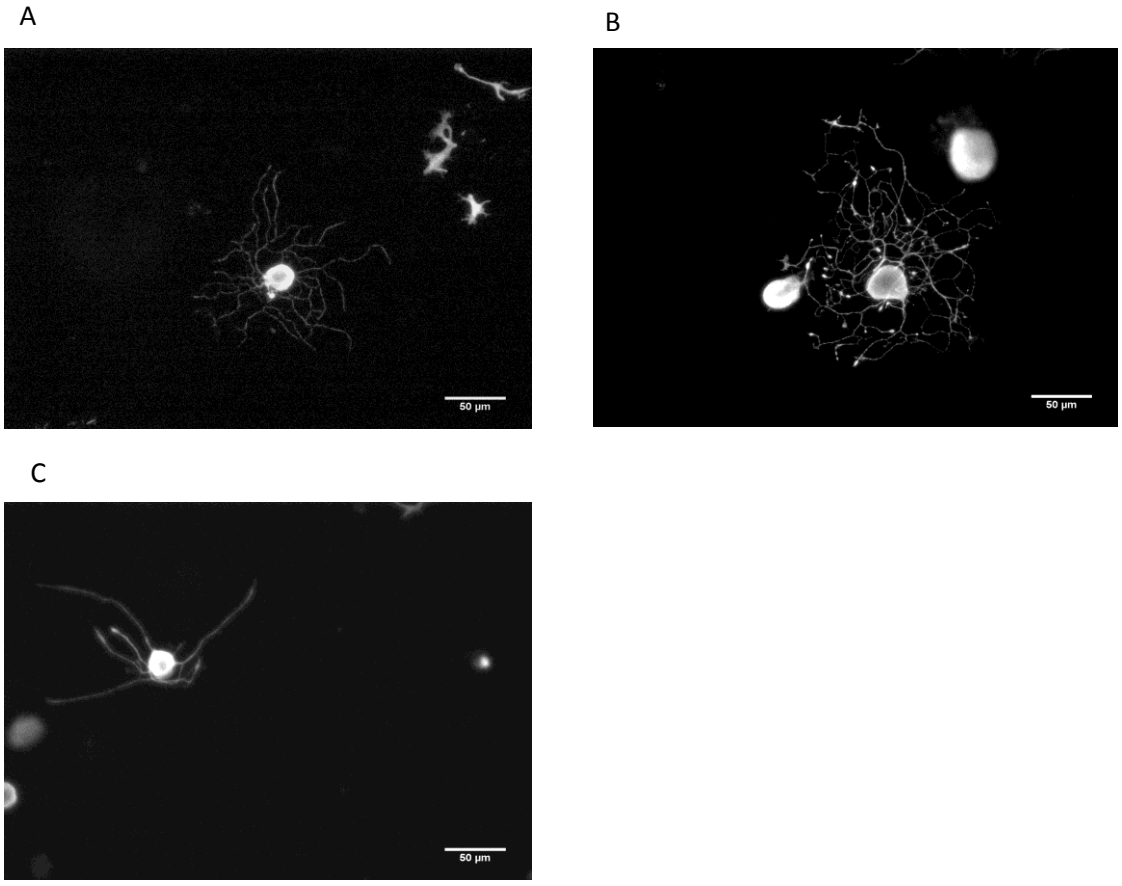


Figure 22: Representative pictures of adult DRG cultures stained for βIII-tubulin. A. DRG cultures in 0,1% DMSO (control); B. DRG cultures treated with inhibitor SB415286 7,5µM; C. DRG cultures treated with inhibitor SB415286 50µM.

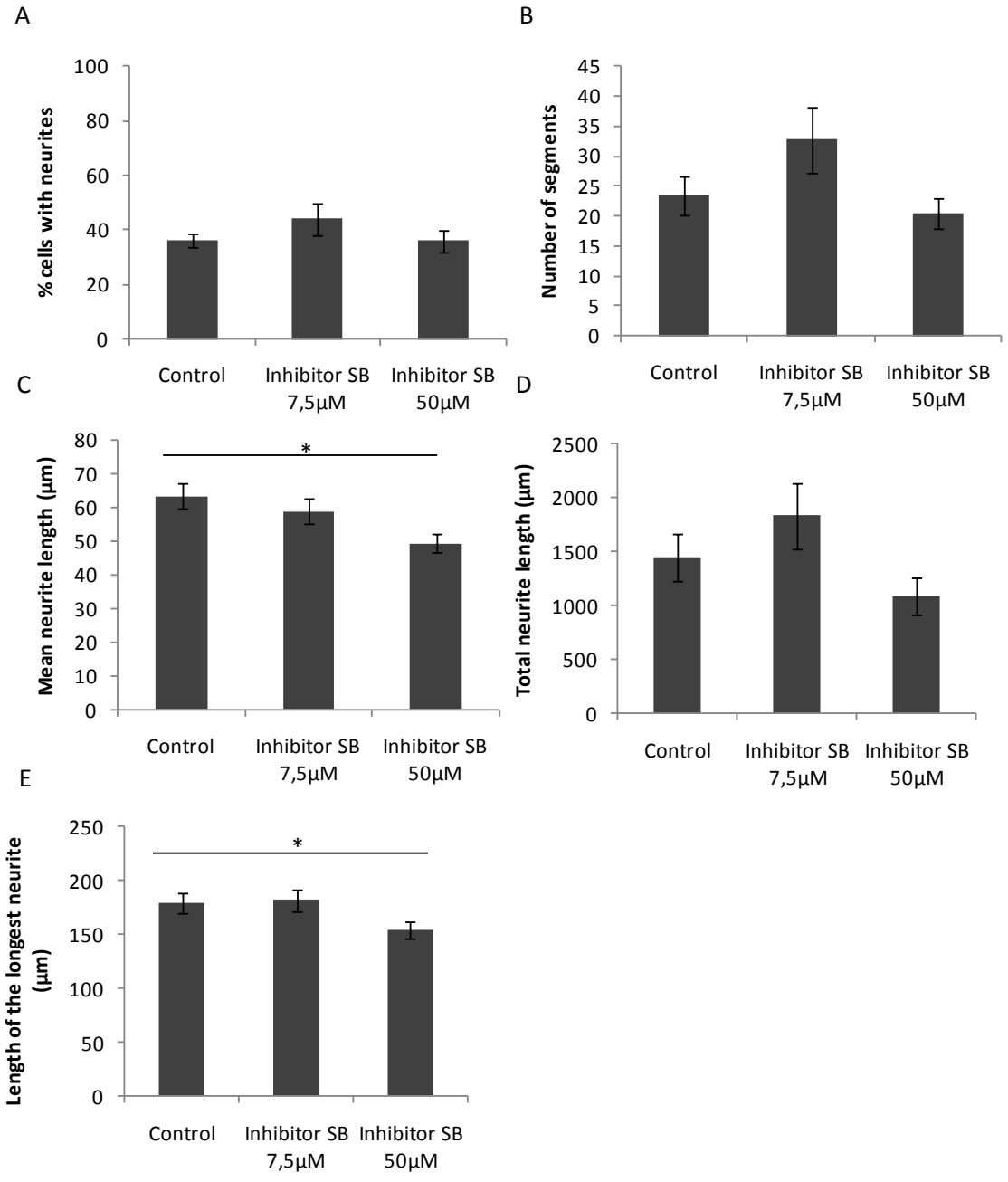


Figure 23: Neurite outgrowth of adult DRG neurons grown in the absence (control) or presence of inhibitor SB (7,5µM and 50µM). The results are expressed as mean±sem. Two-tailed Student's t test with $p<0,05$ (*).

Regarding the inhibitor X, representative images are shown on Figure 24. For this GSK3 inhibitor, no significant differences were found for any of the conditions. The only remarkable exception was the increase of the percentage of cells with neurites for inhibitor X 30nM and the decrease for the inhibitor X 300nM (Figure 25).

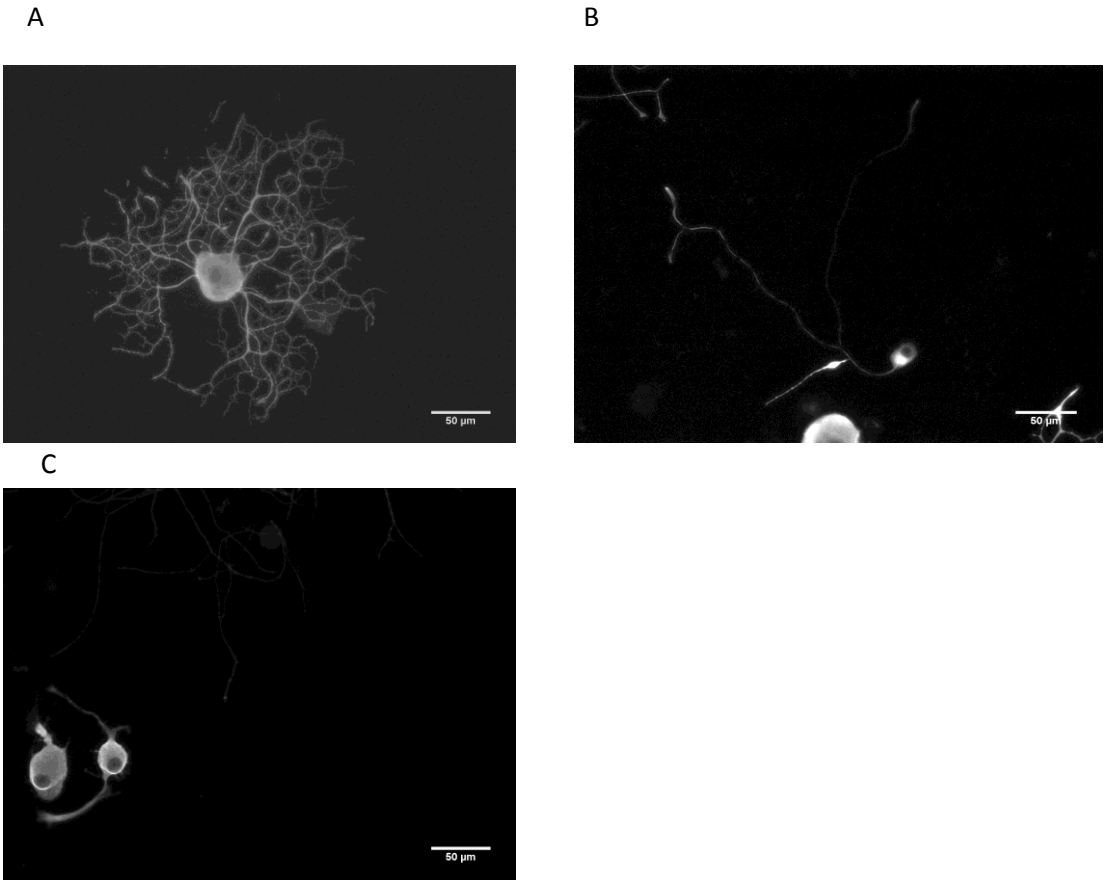


Figure 24: Representative pictures of adult DRG cultures stained for βIII-tubulin. A. DRG cultures in 0,1% DMSO (control); B. DRG cultures treated with inhibitor X 30nM; C. DRG cultures treated with inhibitor X 300nM.

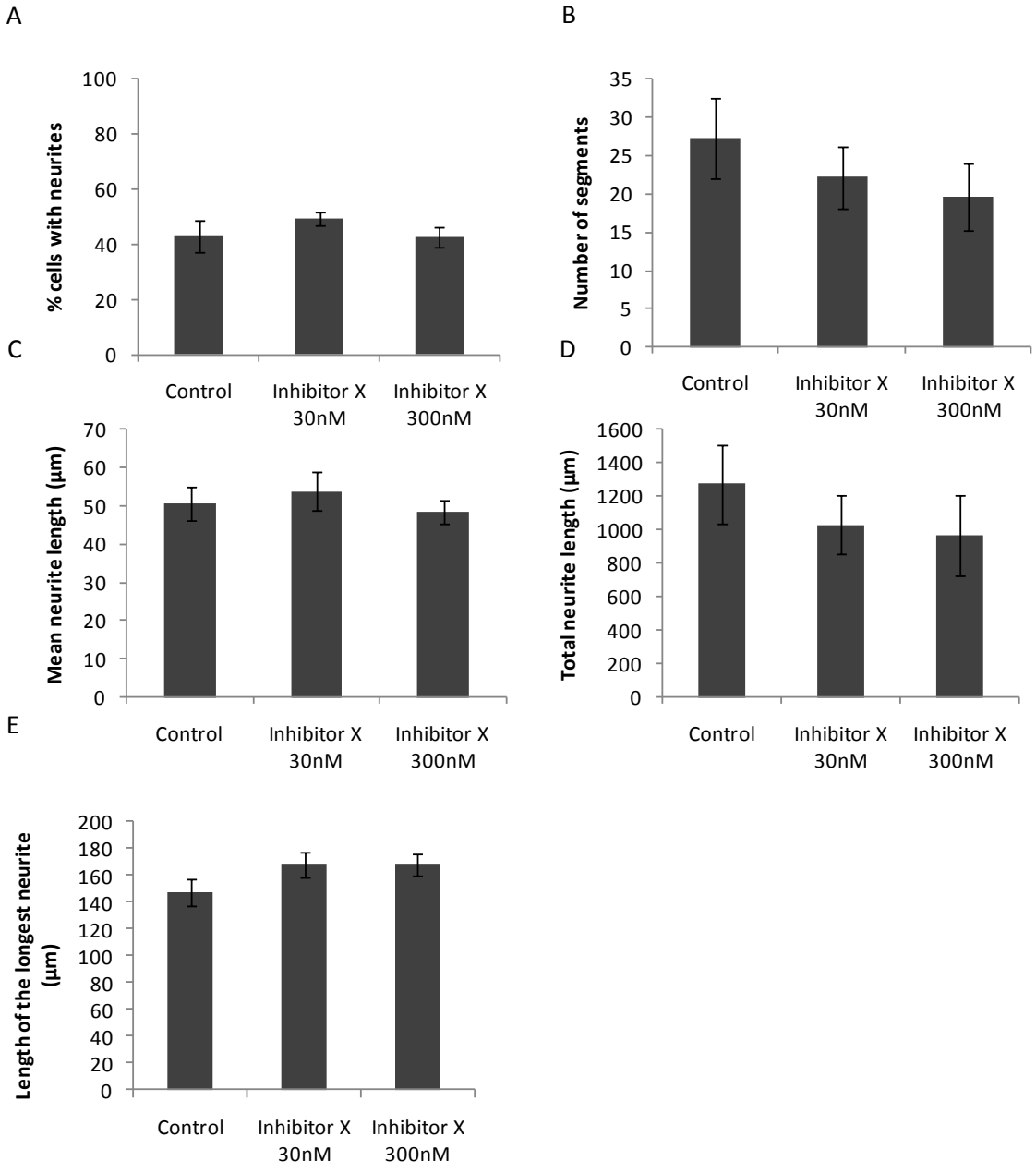
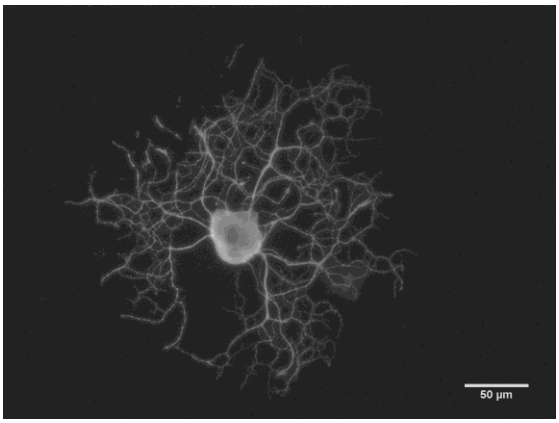


Figure 25: Neurite outgrowth of adult DRG neurons grown in the absence (control) or presence of inhibitor X (30nM and 300nM). The results are expressed as mean±sem. Two-tailed Student's t test with $p<0,05$ (*).

For the inhibitor VII, representative pictures are shown in Figure 26. In this case, the percentage of cells with neurites increases with the lowest dose of inhibitor and decreases with the higher dose, in comparison with the control. In addition, there is a decrease of the number of segments and total length from the control to the highest concentration of inhibitor. On the other hand, the length of the longest neurite is raised

for the lowest dose of inhibitor VII (Figure 27). Our results suggest that inhibitor VII is promoting the conditioning effect (not only by increasing the percentage of cells with neurites but also by increasing elongation). However, in order to confirm this result we will repeat this experiment in the future and we will perform Western blotting to assess the phosphorylation status of GSK3β.

A



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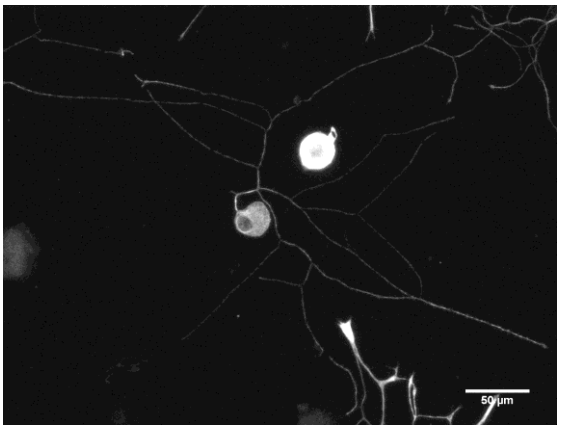

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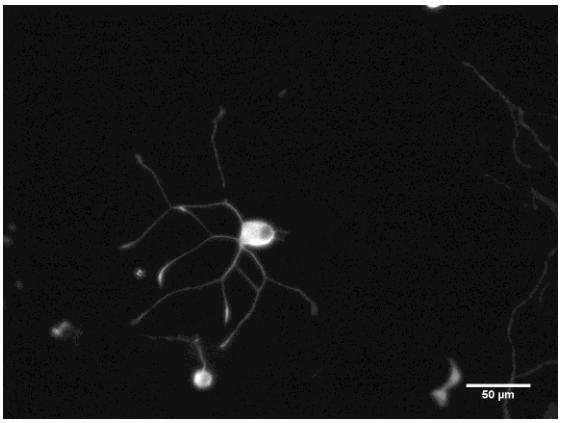

Figure 26: Representative pictures of adult DRG cultures stained for βIII-tubulin. A. DRG cultures in 0,05%DMSO (control); B. DRG cultures treated with inhibitor VII 1µM; C. DRG cultures treated with inhibitor VII 10µM.

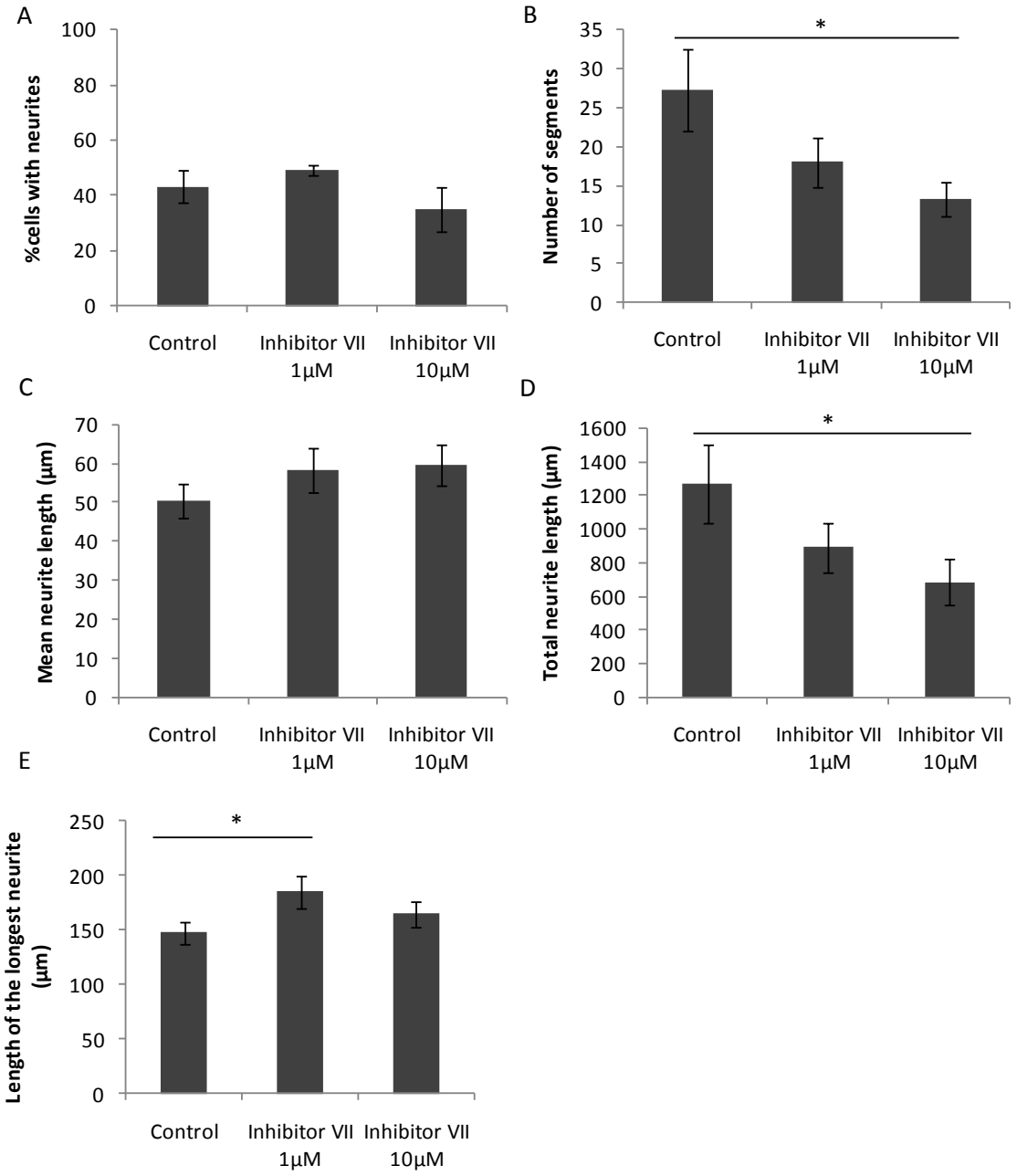

Figure 27: Neurite outgrowth of adult DRG neurons grown in the absence (control) or presence of inhibitor VII (1µM and 10µM). The results are expressed as mean±sem. Two-tailed Student's t test with $p<0,05$ (*).

7. Identification of the mechanism responsible for the modulation of Y216 phosphorylation

The third part of this work consisted on designing strategies to shed light on the kinases responsible for GSK3β(Y216) phosphorylation. Two strong candidates were the tyrosine kinase Pyk2 and Fyn, described in the literature as potential GSK3β regulators by Y216 phosphorylation. We performed Western blotting of Pyk2 in extracts both from spinal cord and DRG, and we could not find this kinase in any of them (data not shown). In parallel, we also performed DRG neuron cultures from animals with spinal cord injury transfected with a kinase dead construct of Pyk2 (Pyk2KD). The percentage of cells with neurites is smaller for the Pyk2KD transfected cells. Regarding the length of the longest neurite, this parameter was similar for the control and Pyk2KD transfected cells (Figure 28). We suppose that these results together with the Western blot of Pyk2 indicate that this kinase does not play a fundamental role in GSK3β modulation.

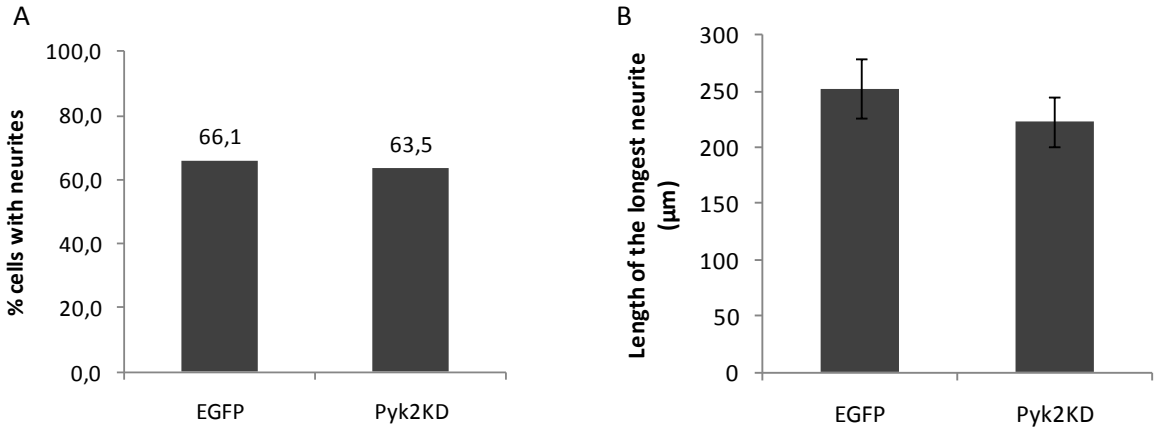

Figure 28: Neurite outgrowth of DRG neurons from animals with SCI transfected with EGFP or Pyk2KD. The results are expressed as mean±sem. Two-tailed Student's t test with $p<0,05$ (*).

In respect to Fyn, our results showed that in the spinal cord, the inactive form of this kinase pFyn(Y529) is increased after conditioning lesion in comparison with SCI only (Figure 29). This suggests that this tyrosine kinase might be a strong candidate for GSK3β(Y216) phosphorylation as an increase in the inactive form of the kinase after CL correlates with the observed decrease in pGSK3β(Y216).

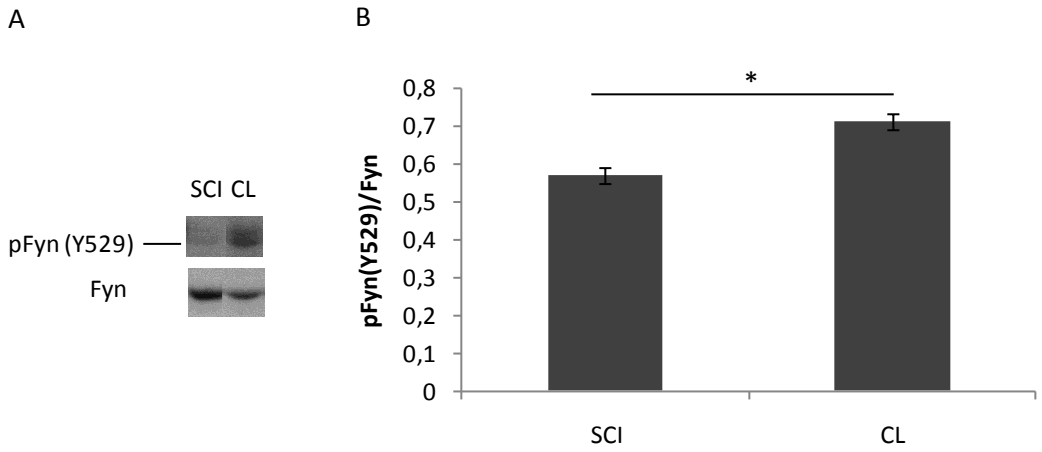

Figure 29: Western blot analysis (A) and respective quantification (B) of the relative amount of pFyn(Y529) in spinal cord extracts of spinal cord injury (SCI) and conditioning lesion (CL) animals. Results are presented as mean±sem. Two-tailed Student's t test with $p<0,05$ (*).

8. *In vivo* analysis of the role of GSK3β in CNS regeneration

Based on our observations, inactivation of GSK3β *in vivo* could mimic the conditioning lesion model and promote regeneration after spinal cord injury. However, as described previously, controversy exists regarding the role of GSK3β modulation in CNS regeneration. While Dill and coworkers consider that GSK3β inactivation is pivotal for axonal outgrowth, Alabed et al suggest that this inactivation through S9 phosphorylation causes neurite retraction instead. In order to better understand the contribution of GSK3β to CNS regeneration *in vivo* we used the GSK3βS9A knockin mouse model. The use of these mice allows us to definitely clarify the role of pS9 in GSK3β regulation of axonal growth. We started by performing neurite outgrowth assays using both uninjured (Un) and conditioned DRG neurons (cDRG). Neurite outgrowth from adult uninjured DRG neurons is short and highly branched, whereas DRG neurons conditioned by prior axotomy *in vivo* are able to extend long and/or un-branched neurites within the first day in culture [60]. To mimetize the conditioning effect we trypsinized DRG neurons after 1 day in culture. In Figure 30, representative pictures for the experiment can be seen. In the case of DRG neurons from WT animals we observed an increase in the percentage of cells with neurites and a significant decrease in the branching after conditioning (WT cDRG). However, contrarily to what is commonly observed in the conditioning effect, no increase of neurite length was observed in the WT conditioned (cDRG) in comparison with the WT uninjured (Un) DRG (Figure 31). In the case of the DRG from GSK3βS9A KI animals, a similar situation to WT animals occurred (Figure 31) which suggests that S9 phosphorylation is not required for the conditioning effect. Moreover, in the case of uninjured DRG neurons no differences in neurite outgrowth were observed between WT and GSK3βS9A KI mice. These results show that regulation of GSK3β activity by S9 phosphorylation is not relevant for the role of the kinase in axonal growth.

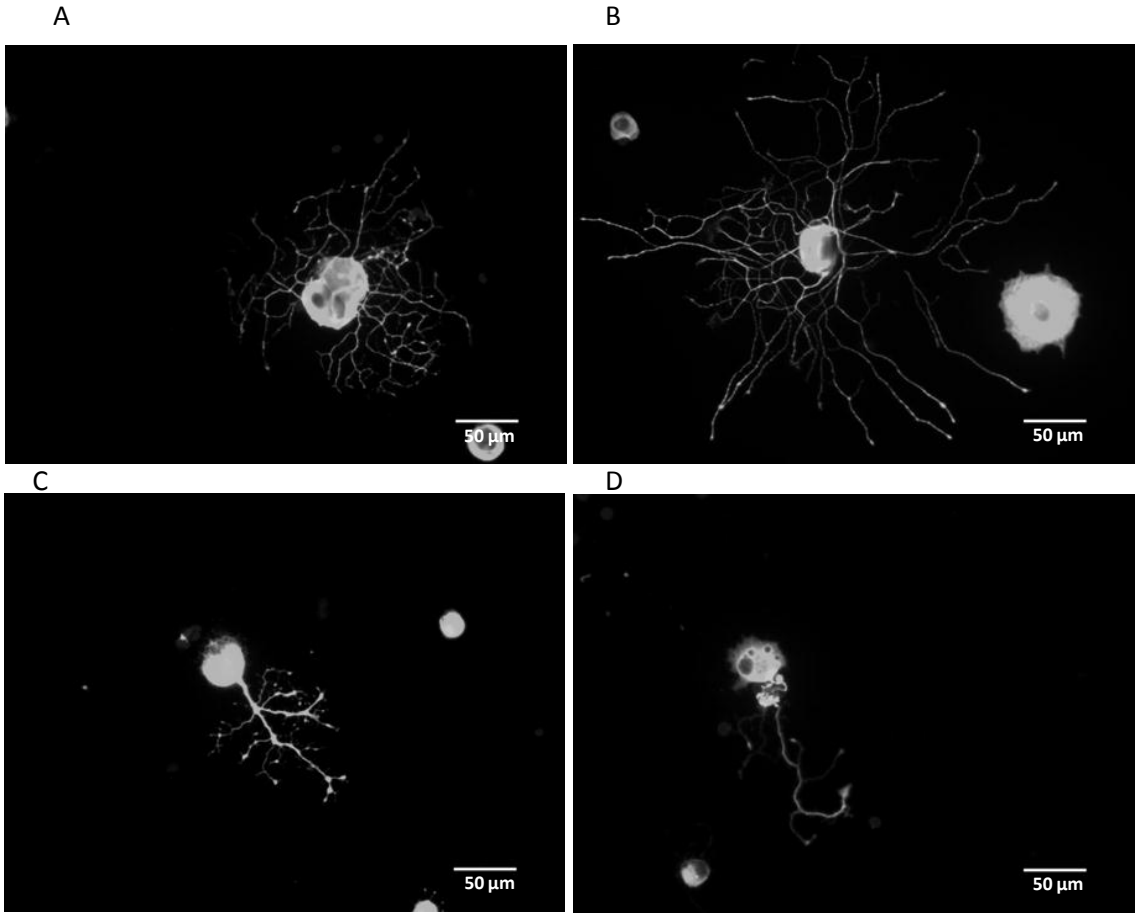

Figure 30: Representative pictures of DRG cultures stained for βIII-tubulin. A. DRG cultures from WT uninjured animals (WT Un); B. DRG cultures treated with trypsin from WT animals (WT cDRG); C. DRG cultures from GSK3βS9A knockin animals (KI Un); D. DRG cultures treated with trypsin from GSK3βS9A knockin animals (KI cDRG).

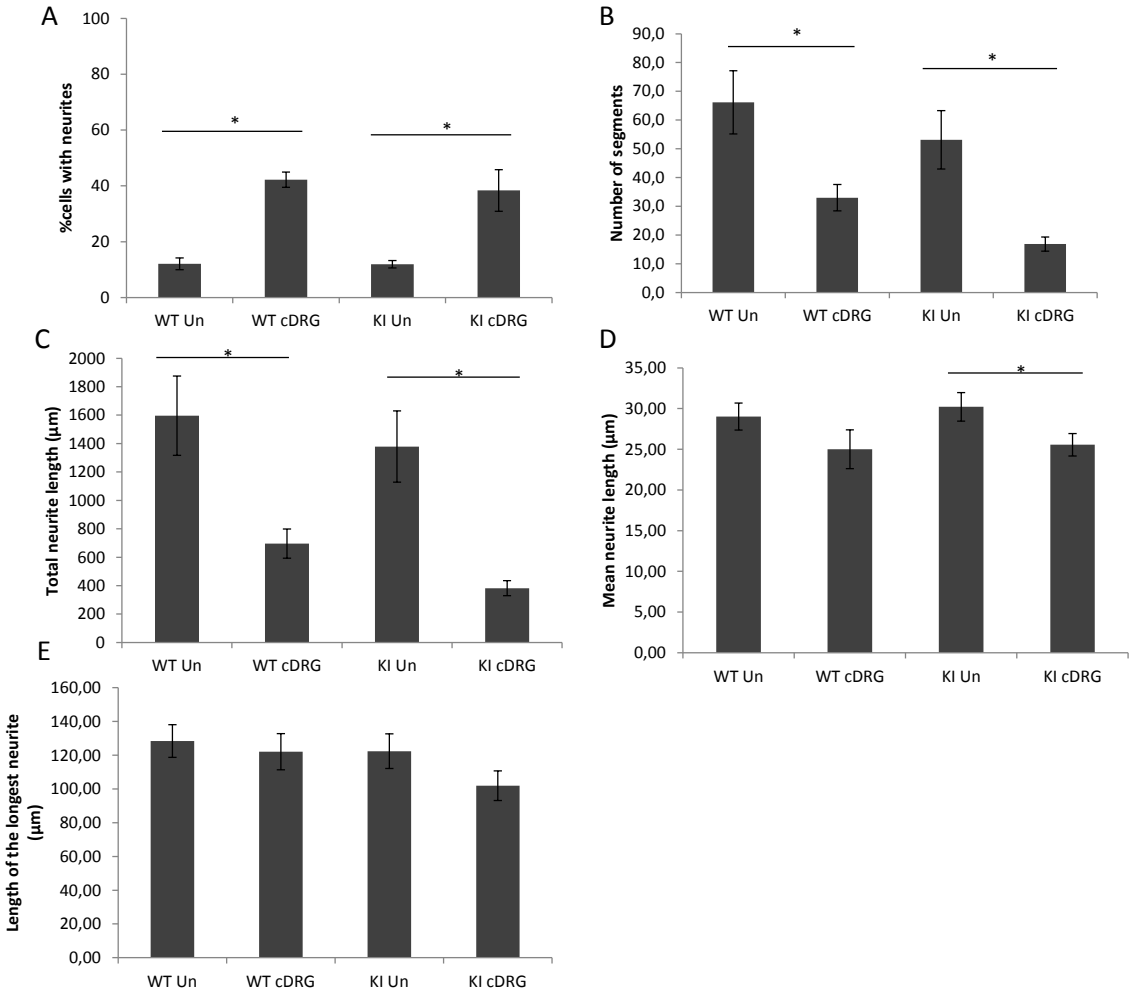

Figure 31: Neurite outgrowth of DRG neurons from WT and GSK3βS9A knockin (KI) animals in the absence (Un) or presence of trypsin (cDRG). The results are expressed as mean±sem. Two-tailed Student's t test with $p<0,05$ (*).

Discussion and Conclusions

It has been described that peripheral conditioning lesion switches DRG neurons to an active growth state, indicating that injury signals traveling retrogradely from the peripheral lesion site to the cell body can enhance the intrinsic growth capacity of the neurons [4]. Previously in our laboratory, two proteomic approaches, Kinexus and iTRAQ revealed several proteins belonging to cytoskeleton remodeling as being differentially regulated following conditioning lesion in DRG extracts. Among those proteins was GSK3β, a serine-threonine kinase which is a key regulatory enzyme in the nervous system. GSK3β emerged as a key player in the conditioning effect, as not only the kinase, but also its effectors and substrates were differentially regulated in the conditioning lesion model. As predicted by our proteomic data and validated by Western blotting, the conditioning lesion triggers a reduction in GSK3β activity in the DRG which might underlie the gain of regenerative capacity in this model.

GSK3β is involved in axonal regeneration locally regulating the cytoskeleton dynamics of the growth cone. In the injury site, after lesion in the spinal cord, a decrease of the levels of S9 phosphorylation of GSK3β can be seen. After conditioning lesion, S9 phosphorylation levels do not show any difference in comparison with spinal cord injury alone. Previously it has been described that after spinal cord lesion the levels of pGSK3β(S9) do not vary in the lesion site but that these levels increase after treatment with the GSK3β inhibitor lithium chloride, leading to a gain of regenerative capacity [3]. Interestingly, in our model, axonal regeneration in the spinal cord is achieved by a decrease in GSK3β activity through downregulation of Y216 phosphorylation promoted by the conditioning lesion. These results point to a regulatory role of tyrosine phosphorylation of GSK3β which has not been addressed in the context of axonal regeneration. In the literature it is mostly described the mechanism of GSK3β inhibition through S9 phosphorylation despite the importance of Y216 residue. Y216 is conserved among all GSK3 homologues isolated so far across very distinct species suggesting a critical role for this phosphorylation [61]. Moreover, some studies doubted the importance of GSK3β(S9) phosphorylation, claiming that GSK3 inhibition regulating

neuronal polarization is not initiated by Akt phosphorylation of S9 [53]. In fact, not only S9 phosphorylation but also Y216 dephosphorylation of GSK3β was shown to be important for the extracellular signal-dependent regulation of GSK3β activity; treatment of Chinese hamster ovary cells overexpressing the human insulin receptor (CHO-IR) with insulin, induced inactivation and tyrosine dephosphorylation of GSK3β both in cells expressing GSK3βWT or cells expressing a truncated form of GSK3β where the N-terminal nine aminoacids were deleted [62].

Based on the decrease observed of GSK3β activity after conditioning lesion we determined the levels of the kinase substrates, CRMP2 and MAP1b, in the spinal cord injury site. In respect to CRMP2, as described in the literature, this protein has two isoforms: CRMP2A and CRMP2B which differ in molecular weight [59]. We observed decreased levels of pCRMP2A(T509/514) after conditioning lesion that can be correlated with the decrease in GSK3β activity. Our results show a decrease in CRMP2 phosphorylation which is not related with an increase in the levels of pGSK3β(S9) but with a decrease of Y216 phosphorylation. Regarding MAP1b, we observed a rise of pMAP1b(T1265) phosphorylation after conditioning lesion in the injury site which, although not in agreement with a lower GSK3β activity, is in accordance with a higher regenerative capacity after conditioning. Our results are in agreement with the hypothesis that inactivation at the growth cone of GSK3β promotes an optimal microtubule assembly for axonal growth by blocking phosphorylation of CRMP2 and promoting phosphorylation of MAP1b [47].

In the DRG, our iTRAQ results showed a decrease in the levels of total CRMP2 (validated by Western blot) and MAP1b. Although this issue was not addressed in this project, this decrease in the DRG may indicate that the proteins are being increasingly transported to the spinal cord injury site after conditioning.

The role for Y216 phosphorylation in the conditioning effect was confirmed by the observation that when conditioned DRGs are treated with the serum-borne lipid mediator lysophosphatidic acid (LPA), which increases the phosphorylation of Y216, the conditioning effect is attenuated which is in accordance with an inhibitory role of pGSK3β(Y216) in axonal regeneration. In fact, it has been described that treating

neuroblastoma cells with LPA causes GSK3β(Y216) phosphorylation and consequently neurite retraction [54]. Consistent with this finding, it was shown that after cerebral ischemia, GSK3β phosphorylation at Y216 is increased in neurons vulnerable to apoptosis suggesting that this phosphorylation site can be an alternative mechanism by which cell insults lead to death [63].

Further analysis of GSK3β modulation through Y216 phosphorylation in axonal regeneration showed us that neurite extension is decreased when Y216 can not be phosphorylated, as is the case of DRG neurons transfected with the EGFP-GSK3βY216F construct. Although this mutant has previously been described as containing a specific activity of 10% of WTGSK3β [61], we observed that overexpression of GSK3βY216F in DRG neurons has the same effect of overexpression of the kinase-dead GSK3βK85M mutant [45]. This in accordance with the fact that GSK3β requires a minimal activity in order to phosphorylate MAP1b.

Taking into account the hypothesis that GSK3β inhibitors treatment might be a better strategy to disclose the role of Y216 phosphorylation in axonal regeneration, we analysed the effect of different GSK3β inhibitors in the neurite outgrowth of adult DRG neurons with the goal to analyse in parallel whether that effect is related to regulation of S9 or Y216 phosphorylation. Several authors tested different GSK3β inhibitors in DRG neuronal cultures but the phosphorylation status of GSK3β was not verified. Adult DRG neurons were treated with two distinct concentrations (low and high dose) for each of three different GSK3 β inhibitors: inhibitor SB415286, inhibitor X and inhibitor VII. The lowest concentration would correspond to a partial inactivation of GSK3β, which seems ideal for neuronal growth, and in contrary, the highest concentration would lead to the enzyme total inactivation that prevents axon growth [45]. For the inhibitor SB415286, no significant difference was obtained in the neurite outgrowth between the control and the lowest dose, unlike Dill and coworkers that observed an increased axon length in the DRG neurons with a low concentration of inhibitor [3]. On the other hand, a decrease of the axonal length can be observed when DRG neurons are treated with the high concentration of inhibitor in agreement with Dill [3]. In the case of the inhibitor X, which shows high GSK3 specificity, no significant differences were seen, contrarily to Kim and his

collaborators that observed a disorder of axon polarity at lower concentrations of inhibitor. They also observed that inactivating GSK3 with a high dose of GSK3 inhibitor causes a decreased axonal regeneration in immature and adult conditioned DRG neurons [45]. Concerning inhibitor VII, we observed an increase in the length of the longest neurite when neurons are treated with the lowest concentration. Regarding the highest concentration, axonal growth is precluded in comparison with the control, which might be due to the total inactivation of the enzyme. This GSK3 inhibitor may be useful for studying the role of Y216 phosphorylation as treatment of cortical neurons with inhibitor VII for 24 hours decreases Y216 phosphorylation, without significant variation in pGSK3β(S9) levels [49]. However, in order to confirm this result, we will need to repeat this experiment and in parallel assess the phosphorylation status of GSK3β.

The third part of this project was intended to unravel the kinases responsible for GSK3β(Y216) phosphorylation. In *Dictyostelium* there is an increase in tyrosine phosphorylation mediated by the ZAK1 kinase. Although no mammalian orthologs of ZAK1 exist [64], three putative candidates are described in the literature: Pyk2 [54], Fyn [65] and Csk [66]. In what concerns Pyk2, this kinase was not found in the spinal cord by Western blot although it is described that Pyk2 is highly expressed in the developing nervous system as well as adult brain [54]. In a model of brain degeneration, Fyn has been associated with Y216 phosphorylation and consequent participation in proapoptosis [63]. Our results suggest that Fyn kinase may be a strong candidate for GSK3β(Y216) phosphorylation, as an increase of the Fyn inactive form pFyn(Y529) is observed after conditioning lesion in the spinal cord. In order to confirm that the decrease of Y216 phosphorylation of GSK3β is due to the inactivation of Fyn, we will analyse whether this kinase co-immunoprecipitates with GSK3β. Moreover, similarly to what was described for the overexpression of GSK3βY216F, transfection of DRG neurons with a Fyn kinase dead mutant will be performed. This will clarify the consequences in neurite outgrowth of downregulating Y216 by inhibiting this tyrosine kinase. Nonetheless, the Y216 phosphorylation mechanism could be an autophosphorylation event as described previously [55]. In fact, in agreement with the autophosphorylation model, it has been observed that a kinase-negative mutant of GSK3 is not tyrosine phosphorylated and on

the other hand, GSK3 produced in *E.coli* is tyrosine phosphorylated [62]. In this case, putative GSK3β phosphatases should be investigated as this might represent the GSK3β regulatory event. Despite the fact that protein phosphatases, including PP1 and PP2A have been implicated in GSK3 serine dephosphorylation [49], so far protein phosphatases capable of dephosphorylating GSK3(Y216) have not been identified.

Finally, we used the GSK3βS9A KI mice [57], where GSK3β activity cannot be regulated by pGSK3β(S9) and as such the role of modulating this particular residue could be definitively elucidated. Our neurite outgrowth measurement results did not show any differences between WT and GSK3βS9A KI animals. According to Dill and coworkers, GSK3β inactivation through S9 phosphorylation leads to increased neurite outgrowth of DRG neurons on myelin and CSPG substrates and stimulates growth of corticospinal tract fibers around the site of SCI [3]. On the other hand, Alabed et al claim that S9 phosphorylation causes axonal growth inhibition in rat cerebellar and DRG neurons [56]. Our results show that regulation of S9 phosphorylation does not have an effect in the growth of adult DRG neurons. Regarding the conditioning effect (obtained by treatment with trypsin), we observed the same effect in both DRG neurons from WT and GSK3βS9A KI mice. Our results show that the conditioning effect may occur independently of S9 phosphorylation. As future work, we plan to repeat this experiment by using conditioned DRGs from animals with axotomy in the sciatic nerve. Also, we will perform *in vitro* neurite outgrowth assays in DRG neurons plated in inhibitory substrates. Moreover, we intend to measure pGSK3(Y216) levels as well as levels of phosphorylated CRMP2 and MAP1b in the GSK3βS9A KI mice. In addition, to assess regeneration *in vivo* in the GSK3βS9A KI mice, we will perform SCI or conditioning lesion and assess regeneration of dorsal column axons by quantification of retrograde tracing with cholera toxin B.

Together our results suggest that GSK3β inactivation (through a decrease in Y216 phosphorylation) leads to axonal regeneration in the spinal cord which is in agreement with several authors [3, 45]. However, as already referred to, there is still some controversy regarding this mechanism, being that some authors claim that GSK3β inactivation leads to axonal retraction instead, through direct S9 phosphorylation by MAIs [56]. This controversy derives mainly from experiments assessing pharmacological

inhibition of GSK3β. In fact, Dill et al showed that treatment of adult DRG neuronal cultures with 3mM lithium chloride or 7,5μM SB415286 enhanced axonal length [3]. On the other hand, Alabed et al observed a decreased neurite outgrowth when treating adult DRG neurons with the GSK3 inhibitor 6-bromoindirubin-3'-oxime (inhibitor X) [56]. We believe that the dose of the inhibitor X used in this study (300nM) provoked the complete inhibition of kinase activity leading to dephosphorylation of both primed and unprimed GSK3β substrates resulting in axonal growth impairment as Kim et al concluded when treating embryonic DRG neurons with high doses of inhibitor [45]. As such, we believe that the difference in results obtained with the pharmacological inhibition of GSK3β may be related to a question of dosage.

As future work we plan to confirm the role of GSK3β in CNS regeneration *in vivo* using additional transgenic GSK3β mouse models namely, GSK3β knockout (KO) heterozygous mice [67], and the floxed GSK3β mice [68]. In heterozygous GSK3βKO mice the kinase activity levels are partially reduced. The floxed GSK3β mice will be crossed with Thy1-cre mice [69] in order to specifically ablate GSK3β activity in axons. The conditional knockout strategy will be used as it was shown that homozygous knockout mice targeting GSK3β die either at mid- or late-embryonic stages with none surviving postnatally [67]. It is important to mention that none of these GSK3β transgenics has ever been used to assess axonal regeneration *in vivo*.

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